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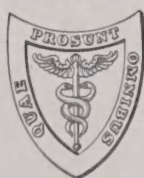
# Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization

Edited By

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*With 71 Illustrations and 130 Tables*



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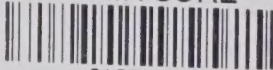
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## PREFACE

THE purpose of this book is to collect, correlate, and evaluate pertinent information relative to antiseptics, disinfectants, and fungicides, as well as chemical and physical methods of sterilization. This has been accomplished by contributions from various authorities who are experts in their respective fields of antimicrobial research. The subject matter covers the most important facts relating to antimicrobial agents generally, exclusive of antibiotics and chemotherapeutic drugs. Because of the diversity of the specialized information involved, the cooperative efforts of recognized authorities make possible reliable and complete surveys of the different phases of this involved and, in some respects complicated array of subjects. More important are the interpretations and evaluations of the subject matter presented by each contributor.

The advice and suggestions of other authorities have been received and incorporated in certain sections. We are grateful to Dr. George Urdang, Director of the American Institute of the History of Pharmacy, for useful suggestions as to the history of germicidal agents. Also, the chapter on definition of terms was submitted to the American Medical Association, American Public Health Association, U. S. Food and Drug Administration, and the U. S. Department of Agriculture for their comments and suggestions. Although these organizations have not given their official approval, there was general agreement, either specific or implied, as to the definitions presented.

Because of different usages of certain terms in the literature and in many text books, especially relating to the words "antiseptic" and "disinfectant," it must be emphasized that current meanings are employed throughout this treatise. At the present time the noun *antiseptic* has a double meaning: a substance which kills bacteria or prevents their growth according to the character of the preparation or the method of application, whereas the word *disinfectant* refers to a chemical substance which destroys infectious microorganisms. Although current usage limits the term *antiseptics* to drugs applied to living tissue and *disinfectants* to chemicals applied to inanimate objects, there are occasions when their use is not strictly confined in this manner. Since there is no verb for the word "antiseptic," it continues to be quite proper to describe the use of germicides on the skin as disinfecting the skin. On the other hand the term "skin antiseptic" is preferred to "skin disinfectant."

On account of the importance of methods of testing antimicrobial agents, this subject is discussed in considerable detail and current procedures are described in separate chapters. Evaluations of various methods are discussed and interpretations of acceptable procedures are presented. Laboratory and practical tests are described in some detail, with appropriate emphasis on each. The necessity and usefulness of both *in vitro* and *in vivo* testing are emphasized and the specific methods of tests are evaluated according to their respective purposes.

The editor is especially grateful to the Board of Trustees of the United States Pharmacopeial Convention for permission to reprint in full the U.S.P. sterility tests for liquids and solids. These methods constitute an important addition to the chapter on methods of testing chemical sterilizing agents. It is useful and convenient to incorporate these tests in a separate section on chemical sterilization.

The grouping of chapters in their respective categories has been somewhat of a problem. Some antiseptics are also used as disinfectants: iodine, for example, which is primarily an antiseptic for use on living tissue, is often used for the disinfection of water. Also some disinfectants such as chlorine, quaternary ammonium compounds, certain phenol compounds, etc. are often employed in antiseptic formulations. Fungicides and fungistats are quite often germicides and bacteriostatic agents respectively. However, the editor has grouped these various antimicrobial agents according to their primary or most common usage, even though there is apparent overlapping in some instances. The classification or grouping used seems most desirable and allows for a certain continuity in the subject matter presented.

The section on chemical and physical methods of sterilization is of great importance for many reasons. Surgical and industrial sterilization are essential processes of considerable public health importance, as is pasteurization. Sterilization by ionizing radiations is one of the newer developments of great interest to the food and pharmaceutical industries as this method is expected to solve many problems.

The editor wishes to thank the various contributors for their interest and willingness to cooperate in the preparation of this book. We are grateful to Dr. Walter Rist of the St. Louis College of Pharmacy and Allied Sciences for his assistance in indexing and preparation of the general bibliographic listing, and to Mr. John F. Spahr of Lea and Febiger for his essential cooperation in the final preparation and organization of the manuscripts. We are also grateful to numerous publishers who have allowed reproduction of photographs, charts, and other copyrighted materials.



It is hardly necessary to reiterate that this book is made possible through the willing and generous cooperation of the contributors. Since the preparation of their respective chapters has required much personal time and effort beyond and in addition to their regular professional activities, it seems quite apparent that their willingness to cooperate is based on an interest bordering on avocation. In this respect their avocation and vocation seem to be one. This is most fortunate since, as so well expressed by Robert Frost:

*"But yield who will to their separation,  
My object in living is to unite  
My avocation and my vocation  
As my two eyes make one in sight.  
Only where love and need are one,  
And the work is play for mortal stakes,  
Is the deed ever really done  
For Heaven and the future's sakes."\**

G. F. REDDISH, *Editor.*

St. Louis, Missouri

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\*Excerpt from "Two Tramps in Mud Time" in A FURTHER RANGE.  
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## PART I

### INTRODUCTION

# I

## HISTORICAL REVIEW

A BRIEF general review of the more important early history of antimicrobial agents and processes may be useful even though further details will be presented relative to specific subjects in the chapters which follow. General information as to the early uses of germicides is often helpful in evaluating subsequent developments. While the intelligent use of germicidal agents covers a period of less than a century, some empirical practices of a useful nature have been employed for many hundreds and even thousands of years. Favorable results were early obtained from practical applications of antimicrobial procedures in the prevention of fermentation and even certain diseases.

There is reason to believe that the early Egyptians considered disease to be contagious and that infection could be transmitted by contact. Specific rules were established by the early Hebrews with respect to the preparation of food, the importance of cleanliness and disposal of waste, preventive measures with respect to leprosy, etc. In fact, many references in the Bible indicate quite clearly an understanding of the effects of bacterial infections. This rudimentary information was known to Aristotle, who advised Alexander the Great to require his armies to "boil the drinking water and bury the dung," a sound military practice based on experience.

It was not until much later that the cause of decomposition and the contagious nature of disease was even suspected. Fracas-



toro (1546) was one of the first to make a clear distinction between decomposition and contagious disease. In a classical publication he grouped diseases as those communicated by contact alone, by fomites, and through the air, and he further indicated that living germs were responsible. Although it was generally known at this time, and for centuries before, that heating food prevented fermentation and putrefaction, it remained for Appert (1810) to develop a really satisfactory method by simply boiling food in sealed containers.

Although at that time there seemed to be no connection between fermentation and disease, there had been an early prediction that the discovery of the cause of fermentation would lead to an understanding of the cause of disease. In 1663 Robert Boyle stated: "And let me adde, that he that thoroughly understands the nature of Ferments and Fermentations, shall probably be much better able than he that ignores them, to give a fair account of divers phenomena of severall diseases (as well as Feavers as others) which will perhaps be never thoroughly understood, without an insight into the doctrine of Fermentation" (Bulloch, 1938). This prophecy was completely fulfilled following the epochal discoveries of Pasteur.

The theory that microorganisms might cause disease had frequently been advanced by certain authorities for many years. In 1840 the famous German pathologist Jacob Henle, in his essay "On Miasma and Contagia," argued for the existence of specific "contagium animatum" for each disease. At that time he suggested three criteria by means of which the causative association could be determined. These required (1) the demonstrated presence of the suspected organism in every case of the disease, but in no other, (2) the isolation of the organism in pure culture, and (3) demonstration that the pure culture was capable of reproducing the disease. These objectives were later attained by Pasteur and Koch who demonstrated beyond question the germ causation of disease. Incidentally, it is of interest to note that Henle in 1840 set forth the three major criteria for determining the etiology of disease which his pupil, Robert Koch used in fulfilling his predictions in the form of Koch's postulates 40 years later.

In spite of the prophecy of Robert Boyle, almost 20 years passed after the cause of fermentation was known before the actual causes of certain diseases were discovered. However, some very significant uses were made of germicides during this twenty-year period, the results of which were highly significant. Although aromatic oils, wine, vinegar, etc., had been used as preservatives for many centuries, solutions of chlorine compounds and carbolic acid were first employed in the nineteenth century, especially during and just

after the controversy on spontaneous generation. Since foul odors were then associated with disease, the first germicides employed were those which were effective deodorants. Labarraque (1825) used chlorinated soda solution in the treatment of infected wounds, and also recommended it for general disinfection. Alcock (1827) introduced the use of this solution into England and recommended its use for the purification of drinking water. At this time it was also used in France for disinfecting the hands and as an important adjunct in the treatment of hospital gangrene. A few years later chlorine solutions were also employed for cleansing the hands as an aid in preventing puerperal fever. Such applications were used primarily for cleansing and deodorizing purposes, and it was not learned until later that the beneficial results were due to destruction of infective microorganisms.

The history of carbolic acid followed a somewhat similar pattern. It was first used as a deodorant on garbage and in sewage to prevent foul odors, and then to prevent wound infections, which were then considered a form of putrefaction. Although carbolic acid was discovered in 1834, it was several years later before it was used in the treatment of wounds. Kuchenmeister (1860) recommended it in place of chlorine solutions as a disinfectant and Lemaire (1860) used it in wounds, the first time pure carbolic acid had been so used, although creosote had previously been employed for the purpose. Later Lister (1867*a*) employed it in treating compound fractures and also in antiseptic surgery (Lister, 1867*b*). Other early uses of carbolic acid in medicine were reported by Bill (1872). It must be noted again that carbolic acid as well as chlorine solutions were used at this time before the actual causes of infections were discovered.

The use of alcohol came somewhat later, although wine had been employed for certain purposes, including preservative and in wounds, for many centuries. Because Koch (1881) had reported that dilute alcohol did not kill anthrax spores by the laboratory test used at that time, the medical profession did not make use of it in their practice, but used mercuric chloride instead which, by the test employed, appeared to be far superior. Later Furbringer (1888) recommended that it be used as an accessory in preoperative preparation to remove fatty material before the germicide to be used was applied; then Reinicke (1894) suggested that in addition to cleansing action 90 per cent alcohol would also be germicidal. Epstein (1897) recommended the use of 50 per cent alcohol and then Beyer (1912) proved quite conclusively the superior germicidal properties of 70 per cent alcohol by weight which is presently used for skin application.

Although iodine had been used in the treatment of goiter as early as 1816, the first specific reference to its use in wounds was made by Davies in 1839, who referred to its curative properties as an external application for the purpose. It is interesting to note that tincture of iodine was admitted to the U. S. Pharmacopeia in 1830 and the compound tincture in 1840, but it was not widely used in surgery until after 1860, or at least results of such use were not reported. It was first used in the treatment of battle wounds during the American Civil War in 1862 when it was employed with marked success. A few years later Davaine (1873, 1875) reported on the bactericidal efficiency of iodine solutions against the anthrax bacillus, and then Sternberg against the gonococcus, and Tarnier and Vignal against staphylococci and streptococci. This was followed by renewed interest in the use of tincture of iodine in surgical practice and especially as a preoperative skin antiseptic, as a result of the detailed and extensive reports by Senn (1905) and Kinnaman (1905), and later by Grossich (1908). During the years from 1910 to 1930 a large series of similar reports was published, including the use of iodine for disinfecting drinking water, used for this purpose since 1904. The proved effectiveness of iodine for these purposes was well established.

It is evident that germicides were at first used for cleansing the hands and treating wounds without any systematic effort being made to find a solution to the perplexing problems of surgical infections. This remained for Semmelweis and Lister who together solved these problems independently in two related fields, puerperal fever and surgical infections. Although not the first to employ germicides for these purposes, these two by their fanatical zeal and careful statistical approach solved these problems, and the intelligent use of antimicrobial agents finally became an established practice. Because of the great importance of the results obtained, Semmelweis by the empirical use of chlorine solution and Lister by logic in the use of carbolic acid, further discussion of their epochal contributions is justified. Since their achievements mark the beginning of a new era in the use of germicides and constitute the foundation stone of modern surgery, a review of their methods and results may be useful.

As early as 1829 Robert Collins used solutions of chlorine compounds in an effort to stop an epidemic of puerperal fever and in 1837 Eisenmann recommended the use of such solutions for the same purpose. Also Oliver Wendell Holmes in 1830 stated his conviction that puerperal fever was transferred from diseased to healthy patients by nurses, and in 1835 reported complete suc-



cess in preventing the communication of such infections by washing his hands in chloride of lime solution after each visit to an infected patient. While it may seem that too much credit has been given Semmelweis for his success in preventing this disease, because of prior use of the same procedure by others, he was the first to use this method in a large lying-in hospital and the first to preserve a careful statistical analysis of his results. Also he published his results in a series of reports over the period from 1847 to 1849, and subsequently in his now famous "Aetiologie" in book form (Semmelweis, 1861). Whereas others had reported briefly on a few cases, he presented conclusive proof based on extensive experience with large numbers of patients.

Semmelweis in 1846 was appointed assistant in the first division ward of the great Lying-in Hospital in Vienna. This ward was used for training medical students, whereas the second division was for instruction of nurses and midwives only. The death rate from puerperal fever was much higher in the first division than in the second, and the only difference in conditions pertaining to each was the fact that medical students would go directly to their ward from the autopsy room adjoining and examine patients and assist in deliveries. Semmelweis noted two significant facts at this time: the lower mortality during the vacation period when the medical students were away and the odor of the autopsy room when they were present in the ward. It seemed apparent to him that there was some direct connection between the students and the high rate of puerperal fever in his ward as contrasted with the rate in the second division where these students were not allowed, and also the fact that the midwives were not permitted in the autopsy room. He immediately suspected the "decomposing organic matter" remaining on the hands of students following a superficial washing with water after autopsies. Since chlorine solutions were known for their cleansing and deodorizing properties, he adopted the rule that no medical student would be allowed to examine obstetric patients or assist in deliveries until they had cleansed their hands with a solution of chloride of lime before leaving the autopsy quarters. The results were spectacular and the incidence of puerperal infections quickly approached that of the second division ward. The death rate in the first division dropped from 12 per cent in May, 1847, before the use of chlorine solution, to 3.0 per cent seven months later, and to 1.27 per cent the following year. This first use of an antiseptic in obstetrics on a large scale marked the beginning of a new era in medicine.

It is of interest to note that Semmelweis became very unpopular among many of the most important surgeons in Vienna as a re-

sult of his achievements, and a great controversy raged between those who supported him and those who opposed. Semmelweis was obstinate and engaged in heated arguments with his detractors, as a result of which he was not reappointed to his position in the hospital, which greatly oppressed him and later led to insanity. Since he was such a controversial figure and because of his insistence that "Murder must cease!", more attention was directed to his results than might otherwise have developed if he had been less obstinate. In contrast, Holmes, on the other hand, a much more dignified and calm personality, simply stated "I will fight with no man over the counterpane which covers a dying mother". It may be that this difference between these two great pioneers is responsible in part for the fact that Semmelweis has received almost entire credit for this important discovery.

Almost 20 years after Semmelweis began the use of chlorine solution in the prevention of puerperal sepsis, Lister began to use carbolic acid in surgery. Although Simpson in England had received a letter in 1848 describing the results obtained in Vienna, apparently this information did not reach Lister, nor was he informed about it when he visited Vienna in 1856. It is quite certain that Lister decided to use carbolic acid in surgery as a result of his own deductive reasoning and not because of information as to prior experience by others. He had long considered that wound infections and gangrene were a form of putrefaction, although the infected tissue was not actually dead. Therefore, when Anderson, professor of chemistry at Glasgow, brought to Lister in 1864 a copy of Pasteur's contribution on putrefaction, published June 29, 1863, he immediately became much interested, especially in Pasteur's conclusion that putrefaction was caused by "living ferments" which were carried on particles of dust in the air. Here at last was the missing link which Lister had been searching for, the reason why wounds "putrefied," and possibly the answer to Robert Boyle's prophecy made 200 years previously. Therefore, Lister reasoned, suppuration in wounds could no doubt be prevented by "applying as a dressing some material capable of destroying the life of the floating particles."

Although Savory (1867) in reporting on the treatment of pyemia stated "Among the most useful of these (treatments) are . . . solutions of carbolic acid, chlorine, chlorinated soda, . . . or iodine," Lister was apparently unaware of these studies when he began the use of carbolic acid in the treatment of compound fracture, the results of which he published in the same year (Lister, 1867a). Instead he relied on his own deductive reasoning that gangrene was a form of putrefaction, that putrefaction was now



known to be caused by living ferments, and that carbolic acid was known to be effective in preventing putrefaction of garbage and sewage, and therefore it might also prevent what he termed the putrefaction of wounds. Because of the high mortality from compound fracture, approximately 50 per cent at that time, Lister decided to test his new theory on these wounds in the spring of 1865. He dressed the wounds with lint soaked with carbolic acid, at first in concentrated solution and later in 1 to 40 concentration, and also disinfected the instruments in 1 to 20 solution. The results were most gratifying, and after further experience in perfecting the technique, infections of this character were brought under complete control. The next step was the use of carbolic acid solutions in general surgery, especially in preoperative preparation and as an application during operations. Also a few years later (1870) he began the use of carbolic acid spray to kill the "floating ferments" in the air and, in spite of many accidents, continued its use for fully 15 years. The use of carbolic acid in general surgery was published during 1867 (Lister, 1867b). These two series of papers by an already eminent surgeon received wide attention, and his system of antiseptic surgery was adopted throughout the world. This new use of antiseptics in surgery, which was later followed by aseptic methods, may well be considered the beginning of the intelligent use of germicides, as it was in fact the cornerstone of modern surgery.

This brief review of the early history of germicides is intended only as an introduction. More complete historical background of specific subjects will be given in individual chapters. Reference to past accomplishments is important as background for intelligent evaluation of later information. Chiseled in marble by the main portal of the Archives Building in Washington is the pertinent phrase "What is past is prologue," so this early history is presented in the nature of a prologue.

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## 2

### DEFINITION OF TERMS

IT IS generally agreed that language is subject to growth and that words acquire new meanings and lose old ones as the years pass. While scientific terms are not so flexible as some, they also are subject to change according to common usage. Also many words have more than one meaning and this, too, is true of certain terms applied to anti-bacterial agents and their activity.

For this reason it is necessary to set forth the present meaning of these words, or the most important of them, as they are employed in the sections which follow. This is also important in connection with the language used in the labeling of drugs and chemicals used as anti-bacterial agents. A judicial decision made under the original Food and Drugs Act states "Language used in the label is to be given the meaning ordinarily conveyed by it to those to whom it was addressed." For legal reasons, therefore, the wording used must be in terms understood by those to whom the labeling is addressed. This must be done regardless of the actual derivation of the words, and also, in certain instances, in spite of strictly technical or professional interpretations.

Two examples of this are the present definitions of the words "antiseptic" and "disinfectant." According to the original derivation, antiseptic means "against putrefaction," or to prevent sepsis, and was originally employed in describing substances or processes which prevented putrefaction or decomposition. Also disinfection means to deprive or eliminate infection or, more specifically, pathogenic microorganisms. Common usage has broadened and even changed the original meaning of these words, or at least qualified their usage.

Consequently there has been, and still is, some confusion as to definition of these words, and also certain others commonly used to describe anti-bacterial activity. In some instances certain of

these words are carelessly or loosely used, even though the accepted meaning is clearly understood, as in the use of the word "sterilize." Also shades of meaning are often ignored, as in the use of the word "prophylactic."

Because of the importance of the proper use of these and similar words in the labeling of antiseptics, disinfectants, and related words, Patterson (1932) made a thorough study of several words that belong in this category. Based on a careful study of the literature on the subject, scientific and general, and consultation with bacteriologists, physicians, public health officials, chemical manufacturers, and laymen, Patterson set forth definitions of these words which reflect present usage.\* The results of this extensive investigation form the basis for some of the definitions set forth here and used in the sections which follow.

### ANTISEPTIC

*Antiseptics* are substances which, when applied to microorganisms, will render them innocuous either by killing them or preventing their growth, according to the character of the preparation or the method of application; used especially for preparations applied to living tissue. (Reddish, 1927).

*Antiseptic*—A substance that opposes sepsis, putrefaction, or decay; one that prevents or arrests the growth or action of microorganisms, either by inhibiting their activity or by destroying them; used especially of agents applied to living tissue. (Patterson, 1932).

The representation of a drug, in its labeling, as an *antiseptic*, shall be considered to be a representation that it is a germicide, except in the case of a drug purporting to be, or represented as, an *antiseptic* for inhibitory use as a wet dressing, ointment, dusting powder, or such other use as involves prolonged contact with the body. (Federal Food, Drug, and Cosmetic Act, 1938).

These three definitions, although worded somewhat differently, are essentially the same and represent the present meaning of the word "antiseptic."

*Comments.*—While the word "antiseptic" is derived from two Greek words meaning, literally, "against putrefaction," and was early limited to this use, the definition has been extended to include sepsis as well as putrefaction and decay. Although sepsis originally meant only putrefaction, it now includes infection by pathogenic microorganisms, so that anything or any substance which opposes sepsis will act against infection as well as putrefaction.

The first known use of this word in English was in 1751 when sea water, myrrh, and acids were described as antiseptics, that is, as substances which opposed putrefaction and decay, which, incidentally, was before it was known that microorganisms caused these conditions. In the early dictionaries and encyclopedias, from

\*See also Webster's New International Dictionary, Unabridged, 2nd ed., 1934.



1819 to 1868, the ability to arrest putrefaction is the only meaning given to this word.

Beginning with Lister's epochal research in surgery, this word acquired an additional meaning, since the germicides used in antiseptic surgery were designated "antiseptics." The American Encyclopedia in 1873 gives these two definitions of the word, (1) to prevent decomposition of organic matter and (2) a substance used to destroy pathogenic microorganisms for use in surgery. As an example, the germicidal solutions of carbolic acid, 1 to 20 and 1 to 40, used by Lister in antiseptic surgery were mentioned.

Since then dictionaries for the laymen and medical profession alike have continued to give two separate and distinct meanings to this word, to inhibit and to kill microorganisms, with the emphasis in recent years to pathogenic bacteria occurring on or in living tissue. For some unknown reason, however American bacteriologists, and especially textbook writers, have continued to use only the original meaning of this word and continue to emphasize the inhibitory definition, and this in spite of the general acceptance of the germicidal meaning by the medical profession and the public.

It is for this reason that this brief history of the word is given here. This is also the reason why it was necessary to formulate a legal definition when the U. S. Bureau of Chemistry (now the Food and Drug Administration) began the control of antiseptics in 1925. At that time Dr. John S. Jamieson, in an address before an association of drug manufacturers, outlined the administrative interpretation of the term "antiseptic" as applied to the control of this class of drugs. This resulted in the legal definition formulated by Reddish (1927) which was used for the purpose until a more precise wording was included in the Federal Food, Drug, and Cosmetic Act (1938).

If a drug acts "against infection," it can do so either by killing infectious microorganisms or by preventing their growth. The most certain means for acting "against infection" is actually to kill the bacteria present. Antiseptics which act in this manner are called germicides. If, on the other hand, a drug is of such a nature, or is used in such a way as simply to prevent the growth of bacteria, and by so doing prevent infection, it can also be classed as an antiseptic. These latter are actually bacteriostatic agents, not germicides. However, they justify being classed as antiseptics because, when used clinically, they act "against infection." It is not difficult, nor is it inconsistent, to give a double meaning to this word. Many English words have double meanings, and the word "antiseptic" is a good example. It must be emphasized that the term is used especially for preparations applied to living tissue, that is, to the living body.



## DISINFECTANT

*Disinfectant*.—An agent that frees from infection; usually, a chemical agent which destroys disease germs or other harmful microorganisms (but not, ordinarily, bacterial spores); commonly used of substances applied to inanimate objects. (Patterson, 1932).

There is little if any disagreement as to this definition. In fact, it is one that has been generally used for many years. However, it is still sometimes misused, such as "skin disinfectant," whereas "skin antiseptic" is more appropriate in describing a germicide for use on the body.

The legal definition employed in the control of disinfectants expresses essentially the same meaning, but is given in more specific detail as follows:

*Disinfectant*.—An agent that frees from infection; usually a chemical agent which destroys disease germs or other harmful microorganisms or inactivates viruses. Most commonly used to designate chemicals that kill the growing forms, but not necessarily the resistant spore forms of bacteria except when the intended use is specifically against an organism forming spores or a virus in which case the spores, too, must be killed or the virus inactivated. Proper use of a disinfectant is contingent on the purpose for which employed or the type of infectious agent that there is reason to suspect may be present.

*Comment*.—Used where the complete elimination of an infectious agent is required such as in the treatment of dishes and utensils from contagious disease wards in hospitals, sick rooms and on equipment, walls, floor, and on farm premises where outbreaks of animal diseases have occurred. Most frequently employed with substances applied to inanimate objects. May be used to sanitize or as an antiseptic. (Stuart, 1952).

*Comments*.—While the verb "disinfect" first appeared in the literature in 17th century, the noun "disinfectant" followed much later. These words were used before the germ theory of disease was advocated and when certain "effluvia" were considered the cause of disease and which could be destroyed by certain chemical substances. Therefore any agent that would free anything from infection was termed a disinfectant. During this time and later disinfectants also were expected to destroy or mask foul odors.

Although the early definitions make no reference to microorganisms, specific germ-killing properties were emphasized following the discovery of the causes of many diseases. Hence it was generally expected that any substance used as a disinfectant would destroy the cause of infection, that is, act as a germicide, and that is the meaning at the present time.

*Note*.—The official definition of the word "disinfection" adopted by the American Public Health Association is as follows: "Disinfection—killing of pathogenic agents by chemical or physical means directly applied." This definition is contained in the American Public Health Association report on "The Control of Communicable Diseases in Man", 7th. ed., 1950, p. 11. It is accepted by the U. S. Public Health Service and also approved by the British Ministry of Health and several other foreign national governments.

Since the word "disinfectant" means, actually, to deprive of or to remove infectious microorganisms, or, more specifically, to destroy them, it has often been used in referring to certain germicides used on the body, such as "skin disinfectants," etc. Even now it is common practice to refer to disinfection of the hands or skin generally by means of germicides, as in pre-operative "skin disinfection." This is not actually a misuse of the term, since the word "disinfect" means just what is implied, yet there has been and is a definite trend to apply the word "disinfectant" to germicides employed to kill infectious microorganisms on inanimate objects. This will, of course, include the inactivation of pathogenic viruses.

While most disinfectants are recommended for use on inanimate objects, in some instances antiseptic uses on the body are also specified. As a practical matter, this cannot be prevented, and laboratory and performance tests for each purpose are employed in the control of such products. At the same time effort is made to encourage elimination of claims for disinfecting as applied to the body and to substitute phrases indicating antiseptic uses instead. Disinfectants should be used only on inanimate objects and claims for such preparations should be made accordingly.

## SANITIZER

*Sanitizer.*—An agent that reduces the number of bacterial contaminants to safe levels as may be judged by public health requirements. Commonly use of substances applied to inanimate objects.

*Comment.*—Used in the day to day control of bacterial contamination with equipment and utensils in dairies and food plants; dishes and glasses in restaurants, taverns and other places where no specific infectious agent is known or suspected to be present, and where the complete destruction of all growing forms of bacteria may not be necessary. To disinfect would be to Sanitize, but to sanitize, it might not be necessary to secure complete germicidal action. Should be restricted to use in connection with cleaning operations. (Stuart, 1952)

*Sanitize* means to render sanitary. The term conveys the idea of disinfection without any residue harmful to subsequent users of the article or product as well as the elimination of any contamination which might be esthetically objectionable. (McCulloch, 1945).

*Sanitizer.*—An agent that reduces the bacterial count to safe levels as may be judged by public health requirements, on food-handling equipment, eating and drinking utensils, and the like. (DuBois, 1949).

*Comments.*—The words "sanitize" and "sanitizer" are now commonly used in the field of public health to denote reduction of bacterial numbers to safe levels as applied particularly to eating and drinking utensils, dairy equipment, etc. It is a less precise word than disinfect and disinfectant, which means to free from infectious microorganisms. The difference is a matter of degree,

but both require rendering the treated equipment safe from transmitting infection. Sanitize includes also a condition of cleanliness, whereas disinfection does not necessarily imply a cleaning process. Also a sanitizer is applied only to inanimate objects, whereas a disinfectant may be and often is applied to living tissue. A detergent sanitizer combines both cleaning and antibacterial properties.

## STERILIZE AND STERILIZATION

*Sterilizer*.—Any process, physical or chemical, which will destroy all forms of life, applied especially to microorganisms, including bacterial and mold spores, and the inactivation of viruses.

*Sterilization*.—The act or process of sterilizing, or freeing from all living microorganisms. (Patterson, 1932.)

There is no disagreement as to the technical definition of these words, although they are loosely applied quite generally by laymen and by the scientific professions as well. These are exact terms and may not be qualified in any way. Also there is no distinction as to the kinds of microorganisms destroyed, whether pathogenic or non-pathogenic, whether vegetative or spore forms, and, of course, includes the inactivation of viruses as well.

Because of the general misuse of these words, the Council on Pharmacy and Chemistry of the American Medical Association (1936) issued a special statement on the subject as follows:

“The Council on Pharmacy and Chemistry has formally gone on record as disapproving of the use of the terms “sterile,” “sterilize” and “sterilization” in a bacteriologic sense other than in their correct scientific significance; *i.e.*, meaning the absence or destruction of all microorganisms. These terms are not relative and to permit their use in a relative sense not only is incorrect but opens the way to abuse and misunderstanding.”

The meanings of certain other terms require no clarification or qualification. For example, the suffix “cide” means a killer or destroyer, and the suffix “stat” or “static” means a condition of inactivity or inhibition or, specifically, prevention of growth. The following definitions, therefore, need not be discussed, except to indicate specific applications required by common usage.

*Germicide*.—Anything that destroys bacteria (microorganisms); applied especially to chemical agents that kill disease germs, but not necessarily bacterial spores; commonly used for substances applied to both living tissue and inanimate objects.

*Bactericide*.—Anything that destroys bacteria; applied especially to chemical agents that kill both pathogenic and non-pathogenic bacteria, but not necessarily bacterial spores; commonly used for substances applied to both living tissue and inanimate objects.



*Fungicide*.—Anything that destroys fungi; applied especially to chemical agents that kill both pathogenic and non-pathogenic fungi other than bacteria; commonly used for substances applied to both living tissue and inanimate objects.

*Viricide*.—Anything that destroys or inactivates viruses; applied especially to chemical substances used on living tissue.

*Sporicide*.—Anything that destroys spores; applied especially to chemical agents that kill bacterial and mold spores; commonly used of substances applied to inanimate objects.

*Preservative*.—An agent or process that prevents decomposition by either chemical or physical means.

There are also adjective and verb transitive variations of these terms which need not be defined since they are self-explanatory, such as germicidal, bacteriostatic, disinfect, disinfection, etc. There are also shades of meaning applied to many of these words according to context.

New words are often coined to describe activities or conditions not described by existing terms. For example, Price (1938) used the word "degermation" to describe the removal of skin bacteria by mechanical cleansing. The same term has become widely used in reporting reduction of bacteria following application of antiseptics to the skin. Other uses of the word may be employed for a variety of conditions, especially following the use of cleansers such as soap and other detergents. As new situations arise and new compounds are developed, it is quite likely that other and different terms will be employed for specific purposes.

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## PART II

### METHODS OF TESTING

# 3

## METHODS OF TESTING

### GENERAL REVIEW

SINCE the bacteriologic methods of testing antiseptics, disinfectants, and fungicides are in general somewhat similar, or at least related, it is desirable to review these methods together. The purposes and general nature of these tests are concerned with the destruction or inhibition of microorganisms. For this reason they may well be considered within the same category.

A review of this subject is best approached from the standpoint of chronological sequence. There are certain obvious advantages in such a procedure since certain of these methods follow a definite order.

While it will not be necessary in this review to devote much attention to the earlier test procedures, it will be of interest to mention them briefly in order to give proper background to the later methods which will be discussed in greater detail. This will be useful also in demonstrating the gradual perfection of present methods of testing based on previous experiences in this field.

### EARLY METHODS OF TESTING GERMICIDES

The first widely used method of testing germicides was devised by Koch (1881) in which silk threads impregnated with spores of *Bacillus anthracis* were subjected to germicides for stated periods of time. Kronig and Paul (1897) modified this method by using

garnets instead of thread. Both these methods gave unreliable results due to bacteriostatic action in the subculture media. They also lacked definite specifications of test conditions and exact means of measuring germicidal activity.

The need for a reliable and exact method for determining germicidal activity was met by Rideal and Walker (1903). This was the first really precise standardized method designed specifically for testing disinfectants and is the basis for all such test procedures now in use. For the first time, important details were controlled and specified in the test.

Anderson and McClintic (1911) modified the Rideal-Walker Test in certain particulars and the resulting procedure became known as the Hygienic Laboratory Method (1912; 1921). This method was widely used in the United States for many years. A special committee of the Laboratory Section of the American Public Health Association (1918) further modified the Hygienic Laboratory Method but this test was not generally approved and has not been widely used.

### IMPROVED TEST METHOD

A few years later Reddish (1927*b*) in cooperation with L. P. Shippen developed a method of test which combined the best features of both the Rideal-Walker Test and the Hygienic Laboratory Method. This method has been widely used for testing disinfectants and in 1931 was designated the U. S. Food and Drug Administration Method (Circular 198, 1931). The Association of Official Agricultural Chemists adopted it as the official method of testing disinfectants (1950).

This briefly is the history of the bacteriological methods of testing disinfectants up to the publication of the U. S. Food and Drug Administration Method and its subsequent adoption as the test procedure recognized and recommended by the Association of Official Agricultural Chemists. These early methods are also of interest in connection with the testing of antiseptics, as will be discussed later. A detailed analysis and evaluation of the later methods may be useful.

Since methods of testing disinfectants were developed first, and then for antiseptics and fungicides they will be discussed in that order.

### PHENOLIC DISINFECTANTS

Although the Rideal-Walker method of testing disinfectants was developed almost half a century ago, it is still being used in most parts of the world as a standard procedure. This being the

case, some consideration must be given this method in a review of the subject, although the test is now rarely used in this country.

The Rideal-Walker Test (1903) was the first really scientific test for standardizing disinfectants and is the basis for all of the standard procedures used at the present time. In this test the following factors are controlled: time, age of culture, choice of medium and its acidity, temperature of medication and incubation, control of resistance of test organisms, specification of a distinct species of test organism, proportion of culture to disinfectant, and the use of a definite standard germicide as control.

Although an English method, the Rideal-Walker Test was early adopted in this country and was later published in American scientific journals (Rideal and Walker, 1913; Walker, 1916). Because of certain undesirable features, the Hygienic Laboratory Method (1912, 1921) largely replaced this test in the United States, and it is now rarely used.

The deficiencies of the Rideal-Walker Test have been recognized for many years and have been discussed in various publications (Moore, 1926; Reddish, 1927*b*; Brewer and Reddish, 1929; Brewer and Ruehle, 1934; Thaysen, 1938; and others). It is the consensus of these authors that the Rideal-Walker Test is incomplete in several respects, such as variability in resistance of the test culture, the necessity of repeated tests in order to obtain a final phenol coefficient figure, lack of definiteness on pH of the culture medium, etc.

Although some of these defects were avoided in the Hygienic Laboratory Method, others were included in this test which made it even more objectionable than the Rideal-Walker Test (Walker, 1916; Reddish, 1927*b*; and others). The principal objections were: variable resistance of culture to phenol controls; no standards for resistance of the test culture; improper culture medium, causing weakening of the test organism; complicated technique; use of open medication tubes; use of 15-second transfers, etc., all of which caused inaccurate and inconsistent results.

In 1918, a committee on standardization of disinfectants appointed by the Laboratory Section of the American Public Health Association gave as its report a new method for obtaining a phenol coefficient for disinfectants (American Public Health Association Method, 1918). In this report the committee modified the Hygienic Laboratory test in such a way as to obtain, at least theoretically, more accurate results. Since this method has not come into practical use in this country a discussion of the procedure will not be given. However, one suggestion was made which is of interest at this time, namely, that phenol coefficients against a variety of disease-producing bacteria other than *Salmonella typhosa* should be determined.



The majority of bacteriologists who do phenol coefficient tests agree that a phenol coefficient against one organism is not of much value, and for that reason further work on the use of different organisms could be considered in the nature of progress in this field.

Because of the above defects of the then standard methods of testing disinfectants, a modified procedure was devised in which the best features of both these methods were retained and the objectionable features eliminated (Reddish, 1926, 1927*b*). In addition to correcting these defects, new features were included for the purpose of making the method more complete. It was suggested (Reddish 1925, 1926, 1927*b*, 1928*a*, 1929) that representatives of the various groups of pathogens should be used as test organisms for determining the germicidal efficiency of disinfectants. The organisms suggested are:

*Salmonella typhosa* (representative of the Gram negative, non-spore-forming bacilli)

*Micrococcus pyogenes* var. *aureus* (representative of the suppurative group, and also Gram positive cocci)

*Corynebacterium diphtheriae* (representative of granular Gram positive groups and diphtheroids)

*Mycobacterium tuberculosis* (representative of the acid-fast group)

*Diplococcus pneumoniae* (representative of the encapsulated, Gram positive cocci)

*Streptococcus pyogenes* (representative of the septicemic pathogens, scarlet fever organisms, erysipelas, etc., Gram positive chain-forming cocci)

Definite phenol resistance standards for the above test organisms were established (Reddish, 1925, 1926, 1927*b*). These standards of resistance have not, however, been widely used, except those for *S. typhosa* and *M. pyogenes* var. *aureus*. Because of renewed interest in the use of various pathogens as test organisms in the study of germicides (Ostrolenk and Brewer, 1949), specific mention of these previous standards is appropriate.

As indicated previously, this is a test of the germicidal activity of disinfectants as compared to pure phenol. The method is limited to phenol-like compounds, that is, disinfectants chemically related to phenol. As stated by the Association of Official Agricultural Chemists (1950) it is "Applicable to testing disinfectants miscible with H<sub>2</sub>O that act against bacteria in manner somewhat comparable to phenol and that do not exert bacteriostatic effects that cannot be neutralized by one of 3 subculture media specified." It is most important that this be clearly understood because considerable confusion has resulted from misuse of the test. The literature on dis-



infectants has been confused by past misuse of the phenol coefficient test and consequent misinterpretation of the results obtained.

This matter has been the subject of much discussion for many years. It has long been agreed that "No single method can serve as a means comparing the value in practice of disinfectants of greatly diverse composition and destined for a variety of application," (Hygienic Laboratory Method, 1921). This same thought has been expressed repeatedly and yet the phenol coefficient test is still being misused. It is being used for compounds not related to phenol and which do not act against bacteria in a manner similar to phenol. For this reason, the matter warrants further consideration.

Strange as it may seem, even mercury compounds and other highly bacteriostatic germicides have been, and some still are, tested by the phenol coefficient method. Shippen (1928) was one of the first to point out the fallacy of using this method for testing such highly bacteriostatic compounds. Later Reddish (1935, 1937) discussed fully the meaning of the phenol coefficient, its significance, and limitations in a thorough study of this matter. Misuse in the case of pine oil emulsions has also been emphasized (Shippen and Griffin, 1923, and others). With respect to quaternary ammonium compounds the fallacy of using the phenol coefficient technique for determining germicidal activity of this class of germicides has been shown (Reddish, 1946). By this time there should be general agreement as to the limitations of the phenol coefficient test, but unfortunately this has not been accomplished.

As a practical matter, the phenol coefficient figure, when obtained on phenol-like compounds, has very important uses. For example, it is widely employed for calculating the dilutions of such disinfectants which may be effectively used in practice (Circular 198, 1931; Varley and Reddish, 1936; Reddish, 1938). It has been proved that when disinfectants chemically related to phenol, which act against bacteria in a manner similar to phenol, are diluted to 20 times their phenol coefficients they are as effective under practical conditions of use as 5 per cent phenol. Experience has proved the efficacy of 5 per cent phenol as a disinfectant, and of phenol-like disinfectants diluted to equal 5 per cent phenol.

The disinfecting value of 5 per cent phenol in controlled tests under somewhat exaggerated conditions of use, was confirmed by Varley and Reddish (1936). At the same time, representative examples of coal tar and cresolic disinfectants having phenol coefficients ranging from 2 to 20 were submitted to similar practical tests in dilutions of 20 x the phenol coefficient of each. The following flooring materials were used in these practical tests: varnished wood, linoleum, enamel, rubber, and asphalt tile, as well as glass

and tin. When applied under practical conditions of use with added exaggerated numbers of *M. pyogenes* var. *aureus* and *Escherichia coli* all of the organisms present on these test materials were killed. Out of a total of 63 tests complete disinfection resulted from the application of the test disinfectants and 5 per cent phenol, usually within 5 minutes, and with two exceptions within 20 minutes. All of the organisms present on these test materials, including those added, were killed by these phenol-like disinfectants when diluted to 20 times their phenol coefficients. This is further proof that the factor "20 times phenol coefficient" is a suitable means for calculating the dilution of phenol-like disinfectants for use under practical conditions.

The results of phenol coefficient tests can be used for four main purposes:

1. To compare the germicidal activity of the disinfectant with that of pure phenol.
2. To compare the relative germicidal efficiency of different phenol-like compounds to determine which are best and most economical to use.
3. To indicate the germicidal properties of disinfectants by a single figure for specification purposes and for ease in purchasing by large agencies such as federal, state, and municipal governments.
4. To use as a means for calculating the effective dilutions for use in practice.

There is a considerable margin of safety in use of the phenol coefficient test. For example, although a disinfectant may kill *S. typhosa* by this test in a dilution of 1 to 450 in 10 minutes, the dilution used in practice would be 1 to 100 and remain in contact with bacteria for much longer than 10 minutes. Also, the dilution calculated from 20 times the phenol coefficient will kill all kinds of pathogenic bacteria except spores. There are a few exceptions to this general rule as shown by *in vitro* tests, but the margin of safety is such that under actual conditions of use the rule is sound. If it is recognized that the phenol coefficient test is limited to disinfectants intended for use on inanimate objects and to those chemically related to phenol, no confusion need arise.

While most bacteriologists interested in the study and testing of disinfectants are fully aware of the limitations of the phenol coefficient as a measure of germicidal activity, it is important that all, and especially teachers, become fully informed in this regard. Much misinformation has been and still is presented to students relative to this important subject.

As has been indicated, there are many kinds of disinfectants which cannot, or rather should not, be tested by the phenol coefficient method. They will be discussed separately. The following table is of interest in this connection.

TABLE 1.—PHENOL COEFFICIENTS

Compound	Method	Temperature Deg. C.	Values obtained with following test orgms.	
			<i>S. aureus</i>	<i>S. typhosa</i>
Chlorine Group				
Chloramine	Rideal-Walker	37	133	100
Dakin's Solution	A.P.H.A.	20		0 78
ICl <sub>3</sub> (0 5% aq. soln.)	A.P.H.A.	20		2 40
Phenolic Group				
Lysol	F.D.A. (Reddish)	20		5 0
Lysol	F.D.A. (Reddish)	20	3 2	
Lysol	F.D.A. (Reddish)	37	4 6	
Creolin	Rideal-Walker	37		9 10
Kreso-Dip	F.D.A. (Reddish)	20		6
Tricresol	(3)			2 6
Ethyl phenol	Rideal-Walker	25	6 0	7 4
n-Propyl phenol	Rideal-Walker	25	16 5	21 6
n-Butyl phenol	Rideal-Walker	25	50	68
n-Amyl phenol	Rideal-Walker	25	139	197
n-Hexyl phenol	Rideal-Walker	25	375	500
Naphthol	Hygienic Laboratory (Antiseptics)	20		11 4
Hexyl resorcinol	Reddish (Modification)	20	150	72
Mercury Compounds				
Mercuric chloride	Reddish	20	143	100
Mercurochrome (220 soluble)	Reddish (Modification)	20	1 7	
Merthiolate (C <sub>2</sub> H <sub>5</sub> HgSC <sub>6</sub> H <sub>4</sub> CO <sub>2</sub> Na)	Reddish	20	40-50	40 50
Metaphen (C <sub>11</sub> H <sub>11</sub> O <sub>7</sub> NH <sub>2</sub> )	Reddish (Modification)	20	1500	
Miscellaneous				
Formalin	Rideal-Walker	37	0 3	0 7
Hydrogen peroxide	Rideal-Walker	20		0 01
Tinct. of Iodine USP	Reddish (Modification)	20	38	
Tinct. of Iodine USP 3% Alcoholic I <sub>2</sub> , USP				
tinct. dil. C <sub>2</sub> H <sub>5</sub> OH	Reddish	20	6 3	5 8
Lugol's iodine	A.P.H.A.	20	5 1	5 0
Menthol	Hygienic Laboratory	20		5 1
Menthol isomers:				
l-Menthol	Rideal-Walker	12-16	5-8	
d-Menthol	Rideal-Walker	12-16	7-12	
dl-Menthol (racemic)	Rideal-Walker	12-16	7-12	
dl-iso-Menthol	Rideal-Walker	12-16	7-12	
Methyl salicylate	Hygienic Laboratory	20		1 76
Picric acid (2 3% in C <sub>2</sub> H <sub>5</sub> OH)	Rideal-Walker	37		6 0

What do these phenol coefficient figures mean and what use can be made of them? What, for example, can be done with Chloramine which has a *M. pyogenes* var. *aureus* phenol coefficient of 135 and an *S. typhosa* phenol coefficient of 100? Chloramine is a chlorine



compound whose chemical and germicidal properties are quite different from those of carbolic acid. Chlorine compounds are unstable and especially so in the presence of organic matter. Phenol is not unstable and is not counteracted by organic matter. A germicidal substance which is counteracted by organic matter such as the chlorine compounds should not be compared with a germicide which is not affected by organic matter. Such a comparison is impracticable and unscientific.

This situation should not exist. Chlorine compounds should not be compared to phenol and phenol-like compounds in the form of a phenol coefficient. In place of a phenol coefficient there should be a "chlorine coefficient" for chlorine compounds, showing the germicidal efficiency of such disinfectants as compared to a standard chlorine solution under specified conditions of a standard test. The present phenol coefficient technique might be employed by substituting a standard chlorine solution in place of the phenol control. Such a test would mean something, and it would be scientifically sound. A "chlorine coefficient" test would not be misleading, and disinfectants tested by this method would not be confused with phenol-like compounds.

In the case of the phenolic group of disinfectants listed in the above table, the *S. typhosa* phenol coefficient figures mean something and can be interpreted in terms of technical and practical values. In the first place, these figures indicate the relative values of these disinfectants for practical use. The purchaser can select these disinfectants with confidence on the basis of the phenol coefficient figures given. When dilutions for use are made on the basis of these figures (20 times the phenol coefficient) the purchaser has the assurance that such solutions will be as effective as 5 per cent carbolic acid under all practical conditions, even in the presence of large amounts of organic matter. Also, the user of such disinfectants has additional protection in the fact that such phenol-like disinfectants are not specific in their germicidal activity and in the dilutions used will kill all kinds of epidemiologic disease-producing microorganisms under practical conditions of use. It has been shown (Reddish, 1935) that there are sufficient margins of safety in such dilutions of phenol-like disinfectants as to assure the killing of all such pathogenic microorganisms under practical conditions of use. Varley and Reddish (1936) proved this to be true with a large number of disinfectants of the coal-tar group, the cresol compound group, and with 5 per cent carbolic acid as well. These phenol-like disinfectants in dilutions twenty times their phenol coefficients were just as effective under practical conditions of use as 5 per cent carbolic acid, a standard germicide which has proved effective under practical conditions of use for over 70 years.



The phenol coefficients of the antiseptics given in the above table are meaningless\* and cannot be used for classifying antiseptics according to their value for use in practice. It cannot be assumed, for example, that Tincture of Iodine which has a phenol coefficient of 38 against *M. pyogenes* var. *aureus* is 7 times better as an antiseptic than is Lugol's iodine solution having a phenol coefficient of 5.1. Tincture of Iodine is more germicidal than Lugol's iodine solution, but not 7 times more effective as an antiseptic than aqueous Lugol's solution under practical conditions of use. The 5 and 2.5 per cent solutions of carbolic acid used by Lister in founding the science of antiseptic surgery have phenol coefficients of 0.05 and approximately 0.02 respectively. These solutions are effective antiseptics, and yet Tincture of Iodine has a phenol coefficient 760 times that of 5 per cent carbolic acid and approximately 1900 times that of 2.5 per cent carbolic acid. Although Tincture of Iodine is apparently 760 times more germicidal than 5 per cent phenol as judged on the basis of phenol coefficient figures of the two germicides, it is not actually 760 times better as an antiseptic.

Many other examples of the impracticability of the phenol coefficient as applied to antiseptics could be given. There is no justification, on scientific, technical, or practical grounds, for the use of this figure to indicate the germicidal efficiency and practical value of antiseptics. Antiseptics have special uses and a phenol coefficient figure cannot be of practical use as an index of their value even for special application. Also, since antiseptics are marketed ready for use or the dilutions specified on the label the phenol coefficient need not—and really cannot—be used for determining the proper dilution for use. Many antiseptics act in a manner quite different from phenol so that any comparison to the germ-killing power of carbolic acid is out of the question. Antiseptics should be tested directly on the bacteria which will be met in practice. This can be done by using the special specific test methods for antiseptics. These tests were designed especially for antiseptics and should always be used for testing this special class of preparations.

Evaluation of results obtained by phenol coefficient tests depends largely on the nature of the germicide tested and the uses for which it is recommended. This cannot be emphasized too strongly since it is most important in any consideration of the subject. The phenol coefficient technique, in use for over half a century for testing disinfectants, is still satisfactory for the testing of phenol-like compounds for use on inanimate objects.

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\* This is not intended as criticism of the Handbook of Chemistry for publishing these figures, but only of those originally responsible for determining phenol coefficients of antiseptics.

## CHLORINE COMPOUNDS

Since there are many germicides which do not act against bacteria in a manner similar to phenol, it has become necessary to develop other methods for testing them. Some of these methods are practical tests, others are tests based on practical use experience. The chlorine compounds, as well as the quaternary ammonium compounds and others, must be studied by procedures quite different from the phenol coefficient method.

One of the early procedures was devised by Tonney, Greer, and Danforth (1928, 1930) for determining the minimal chlorine death points of bacteria. Various pathogenic bacteria were exposed to specified concentrations of chlorine in water for short time periods and plate counts made of the surviving organisms. A large percentage of these strains were killed by 0.15 ppm of free chlorine within 15 to 30 seconds whereas none survived 0.25 ppm. Since *E. coli* was the most resistant, it was used as an "index organism" in these tests, and for use as a criterion of effective chlorination of water supplies, swimming pools, dairy equipment, dishes, and other utensils. The spore-formers, on the other hand, required from 2.5 to 280 ppm for complete killing under the conditions of the test. *Bacillus subtilis* was found best as an "index organism" of the spore-bearing group. Since it is futile, and from a sanitization standpoint unnecessary, to kill the spore-bearing organisms, the primary purpose of this test is served by using only *E. coli* as the "index organism." This is a good test for the purposes designated and reflects accurately the practical disinfecting value of free chlorine and chlorine compounds, when used in water.

Tilley (1920), in studying the germicidal value of chlorine disinfectants, made use of blood serum as organic matter, in order to test the practical value of such compounds. He added the serum to the dilutions of disinfectants before the test organisms were added to them and then transferred a loopful of the mixture to nutrient broth at intervals of 15 minutes, 1 hour, and 2 hours. As a result of this study, he concluded that chlorine compounds should not be used where large amounts of organic matter are present. He also observed that by this test chloramin T is much less effective against *Pseudomonas aeruginosa* than against *M. pyogenes* var. *aureus*.

Mallmann and Gelpi, Jr. (1930) conducted practical tests on the effect of chlorine in water on colon bacilli and streptococci. The test organisms were isolated from swimming pools and then used for determining the germicidal effectiveness of chlorine. Since it was shown that *E. coli* developed a tolerance to free chlorine, streptococci were recommended as test organisms for chlorine com-

pounds used in swimming pools. Here again, the test used for chlorine compounds was essentially a practical test. Later Mallmann and Edwards (1934) showed that bacteriologic tests were necessary to determine the germicidal value of chlorine in the presence of suspended material and that the iodometric and the ortho-toluidine methods for measuring available chlorine do not accurately measure the germicidal chlorine present. These tests also were conducted under practical conditions of use.

On account of the wide use of chlorine compounds, especially hypochlorites, for the disinfection of dairy equipment, milk plant apparatus, restaurant dishes, etc., it is important to know exactly how effective these disinfectants are under conditions of use. One of the organisms of importance in this connection is the tubercle bacillus. Costigan (1936) using suspensions of *M. tuberculosis* in high concentration determined the effect of water solutions of hypochlorites in killing this resistant organism. When exaggerated numbers of this organism were used in these tests, 50 ppm available chlorine killed the tubercle bacillus within 0.5 to 2.5 minutes when the temperature of the solution was between 60° and 50°C respectively. This is a practical determination of the germicidal activity of hypochlorites against a resistant test organism and the results obtained can be interpreted directly in terms of practical value under conditions of use.

Another example of the practical testing of chlorine compounds is that reported by Charlton and Levine (1937). Using suspensions of bacterial spores the degree of germicidal activity of chlorine compounds was studied at varying degrees of concentration, temperature, and reaction (pH). Again it was proved that the "available chlorine concentration of a given compound is not in itself a direct measure of the germicidal efficiency of the compound." The effect of pH is especially noted in this connection, acid compounds being more germicidal than alkaline of the same chlorine concentrations.

Still another example of the practical testing of chlorine compounds and the direct evaluation of the results obtained is a method employed in the study of succinchlorimide as a water disinfectant (Reddish and Pauley, 1945). Previously, the properties of a water disinfectant for use by the military establishments had been listed (Reddish, 1943). The specifications were quite severe, requiring the killing within 10 minutes, at temperatures ranging from 36° to 120°F, of all disease-producing bacteria carried by water. Such disinfectants should prove germicidally effective in the presence of excessive amounts of organic matter. The method of test employed for this purpose is more severe than those discussed above.



Heavily polluted sewage with a chlorine demand of 15 ppm was inoculated with large numbers (up to 1,000,000 per ml) of the following pathogenic intestinal organisms: *S. typhosa*, *Shigella dysenteriae*, *Salmonella paratyphi*, *Salmonella schottmuelleri*, *Vibrio comma*, and *E. coli*. After the succinchlorimide was added to 1 L of water in specified amounts, and held at 36°, 73°, and 80°F, 1 ml portions were plated in nutrient agar at 5, 10, 20, and 30 minute intervals. The plates were incubated at 30°C for 48 hours and counted. All of the organisms present, including those added, were killed within 10 minutes by the dosage recommended for use in practice. Similar concentrations were tested against dysentery ameba cysts by the method described by Chang and Fair (1941) and Chang (1942) and found to destroy them under such conditions with 10 to 20 minutes. Since these tests were conducted under conditions closely simulating practical application under extreme conditions, the results may be interpreted directly in terms of actual use value.

Value in actual use is most important in connection with the testing of chlorine compounds. Furthermore, practical test procedures are necessary since there is no standard test for determining the disinfecting value of chlorine compounds such as the phenol coefficients for phenol-like disinfectants. For the present, at least, this is the only recourse until such a standard test is developed for chlorine compounds.

Somewhat the same situation exists with respect to other non-phenolic disinfectants, in fact for all germicides which do not act against bacteria in a manner similar to phenol. The quaternary ammonium compounds are a good example. Here again, at least for the present, it is necessary to use procedures which simulate practical conditions of use.

## QUATERNARY AMMONIUM COMPOUNDS

It is interesting to note that quaternaries are somewhat selective in their germicidal activity, being more effective against the gram positive cocci than against the gram negative bacilli—just the reverse of the usual activity of most disinfectants. For this reason, laboratory methods of testing should use both kinds of organisms.

Although the phenol coefficient method is not applicable, since these compounds are not phenol-like, the phenol coefficient method employing *S. typhosa* and *M. pyogenes* var. *aureus* as test organisms has been widely used. Even when both organisms are used the test is still unsatisfactory. Irregular results are obtained, with “wild



pluses" appearing in a most unexpected fashion. For this reason it is necessary to perform a large number of tests in order to arrive at a reasonably accurate result. Even so, the phenol coefficient figures reported vary considerably among different laboratories, and even when the tests are conducted in the same laboratory. In spite of this, and because there is no acceptable standard method of test, phenol coefficient figures are now being used for comparative purposes, for control purposes, and for calculating the dilutions for use in practice, (the 20 times coefficient factor being employed.)

One such quaternary when tested at 20°C has a phenol coefficient against *S. typhosa* of 200 to 250 and against *M. pyogenes* var. *aureus* of 400 to 500. The germicidal dilution against certain organisms, for example *S. pyogenes*, ranges up to 1 to 1,000,000 and the bacteriostatic dilutions up to 1 to 2,000,000 to 1 to 4,000,000. It is evident that such a compound creates problems in laboratory testing. A further complication is the fact that the presence of organic matter adversely affects the germicidal activity of this class of compounds.

In an effort to determine the actual germicidal effectiveness of such disinfectants by practical test, the "Use-Dilution Method" of Mallmann and Hanes (1945) has met with wide approval. This method makes use of small glass rods on which the test organisms are dried for 30 minutes. The seeded rods are then exposed to the test solutions at 20°C for 1, 5, 10, and 30 minutes, rinsed with water or neutralizing solution, and transferred to subculture media. While this method was not specifically designed for quaternaries, it has been found quite suitable for the purpose. In fact, when quaternaries which satisfactorily pass this test were also tested under practical conditions of use, they were found satisfactory for disinfecting eating and drinking utensils, etc. Although care must be exercised in conducting this test, especially in drying the test cultures on the rods, it is satisfactory for the purpose when properly employed.

Mallmann, Kivela, and Turner (1946) conducted extensive comparative laboratory tests on hypochlorite and quaternaries by the phenol coefficient method, the Use-Dilution Method, a wet filter paper technique, and in addition used a new procedure designated the "Speed Reaction Test." Results obtained by these tests were compared to practical field testing using hundreds of beverage glasses under use conditions. Such practical test methods should be employed for the testing of quaternaries recommended for the disinfection of eating and drinking utensils.

Whether or not they will become standard methods depends upon future experience in their use. Mallmann and Leavitt (1948)

after a rather extensive study of the Use-Dilution Method, as compared to the phenol coefficient method, have come to the conclusion that the Use-Dilution Method is quite satisfactory even for testing phenol-like compounds. In fact, they find the Use-Dilution Method superior to the phenol coefficient method, especially for disinfectants which exhibit high phenol coefficients. While results obtained by the Use-Dilution Method are lower than by the phenol coefficient procedure, when this figure is multiplied by 20, as is required, the dilution for use in practice is then quite comparable to results obtained by the Use-Dilution Method. It is important to re-emphasize that the results obtained by the Use-Dilution Method are interpreted directly into dilutions for use in practice, whereas the figures obtained by the phenol coefficient method must be multiplied by 20 in order to secure the dilution for practical use. For this reason, there is no necessity for changing from the present method for testing phenol-like compounds. As shown earlier, this method is satisfactory for the purpose.

Klarmann and Wright (1946) made a rather extensive study of the germicidal activity of several disinfectants representing both phenol-like compounds and quaternary ammonium compounds. A modified technique was used by simply employing one-tenth the quantity of disinfectant dilutions and culture (0.5 ml of disinfectant dilution and 0.05 ml of test culture). After mixing in large medication tubes, broth (20 ml) was added to the medication mixture at stated time periods (5, 10, and 15 minutes). This modified procedure is referred to as a "semi-micro" method.

Results by this "semi-micro" method, as might be expected, are somewhat lower when used on phenol-like disinfectants than those obtained by the usual methods since 25 times the inoculum is used in the subculture media. However, and this is the most important result of this modified technique, the results obtained with quaternaries are very considerably less than by the previous method. In discussing the possible reasons for this, it is suggested that the bacteria in the medication tubes are attracted to the side walls of the tube under the influence of the quaternaries, in which case they may be missed by a 4 mm loop transfer but not by the "semi-micro" technique. While this theory may seem plausible, it has not as yet been proved, whereas the work of certain others seems to disprove it. Regardless of the reasons for this phenomenon, it is established that more unfavorable results are obtained with the "semi-micro" technique on quaternaries than by the other methods.

The question naturally arises as to significance of the difference in results obtained by the two methods. In the first place, the use of even a modified phenol coefficient method for testing quaternaries

and other non-phenolic disinfectants has been criticized for the simple reason that they do not act in a manner similar to phenol. As a result of use of this modified phenol coefficient test, it is indicated that quaternaries are less effective germicides than phenol-like disinfectants.

From a practical standpoint this is of only academic interest. The important consideration is "Will quaternary ammonium compounds effectively kill bacteria under practical conditions of use?" The laboratory method of testing should be based on results obtained under actual conditions of use. Instead of using the phenol coefficient method of test, or modifications of it, and then attempting to interpret these results in terms of practical values, the results of practical tests must first be determined and laboratory tests then be devised accordingly.

Our present methods of testing disinfectants, as stated above, are all based on practical tests under conditions of use. There is no reason for making an exception in the case of quaternary ammonium compounds. Since the phenol coefficient test should not be used, and since it is impossible to interpret the results of such tests, a laboratory method must be adopted which can be interpreted in terms of practical values.

The laboratory test should be a practical test, a "use-dilution" test which simulates practical conditions. Such a method is already available, the "Use-Dilution Method" developed by Mallmann and Hanes (1945). While this method is recommended for testing disinfectants generally, it seems to be especially suitable for quaternary ammonium compounds.

Under actual conditions of use, a 1 to 1000 dilution of quaternary ammonium compound employed in dishwater sanitation reduced the number of bacteria on drinking glasses from an average of 715 to an average of 1.75 per rim, and reduced the number of bacteria in the rinse water from an average of 45,533 to an average of 1.5 per ml (Bartlett, 1946). Such reductions under practical conditions are highly satisfactory and come well within the requirements of the U. S. Public Health Service for safe drinking water. Practical experience with quaternary ammonium compounds for the sanitizing of eating and drinking utensils has given satisfactory results. It is for this reason that the Use-Dilution Method is recommended for the laboratory testing of this class of disinfectants.

Another observation, made by Klarmann and Wright (1946) was that after exposure of test culture to quaternary dilutions some bacteria remained alive by the "semi-micro" technique which had apparently been killed by the phenol coefficient method. Also, it was shown that when a glass strip was placed in the medication



tube some bacteria remained alive that were apparently killed by the "semi-micro" technique when quaternaries were used, whereas this did not result in the case of phenol. Since this result has been confirmed by others it will be of interest to consider the significance of such results in terms of practical value.

In the first place it has been shown by Romans (1942) and others that when no growth is obtained in subculture after loop transfer, either all of the test culture has been killed or only a few organisms have survived. That is, so few remained alive after exposure to the germicide that they did not grow in subculture after loop transfer. However, since exaggerated numbers of organisms are used in the test, the few remaining are of no practical significance. Using the figures of Klarmann and Wright (1946) the number of organisms killed in the test were 99.999+ per cent of the total exposed to the germicide. The results obtained by Romans (1942) were complete kill or 99.999+ per cent reduction in those tests showing no growth by the loop transfer.

Considerable discussion as to the necessity of requiring complete kill in laboratory testing of germicides has developed. The reason for requiring complete killing of test organisms in laboratory tests, presumably, is that when used in practice disinfectants must render the objects treated free from danger of infection. That, of course, is the purpose of disinfection. However, since laboratory tests for disinfectants employ exaggerated numbers of the test organisms, it is not actually necessary to kill all the organisms under these *in vitro* conditions. In other words, a germicide which kills 99.999+ per cent of the test culture is completely satisfactory under conditions of use in which far fewer organisms are present. This is made quite clear by Quisno, Foter, and Rubenkoenig (1947) who showed that although 350,000,000 of *M. pyogenes* var. *aureus* were used in tests on quaternaries, only from 0 to 40 organisms survived after exposure to dilutions ranging from 1 to 8,000 to 1 to 20,000. This would be a reduction of 99.999+ per cent which for all practical purposes is equivalent to complete kill.

This whole matter has been carefully studied by Klimek and Umbreit (1948). First they found no difference in germicidal results when 4 mm loop transfer was compared to 0.05 ml transfer, using a quaternary and phenol. The phenol coefficient of the quaternary was the same by both methods. Then using a broth culture of *M. pyogenes* var. *aureus* containing 431,400,000 organisms in 0.5 ml of inoculum, they compared the results of these tests with the number of surviving organisms at the various dilutions and time periods used in the test, using a quaternary as the germicide. Here again the numbers of living test organisms present in the



medication mixture giving no growth in subculture was insignificant. Actually there was at least 99.999+ per cent kill at time periods showing no growth by loop transfer into subculture broth.

Furthermore, it was shown that the results from tests with a quaternary were the same when the glass strip technique was used as when the "semi-micro" technique was used. It is concluded from this study that the so-called "fallacious" results reported relative to quaternaries are not due to massive adsorption of the test organisms upon the glass walls of the medication tubes.

This whole discussion develops around the question of whether the results obtained by the phenol coefficient method show 100 per cent kill or 99.999+ per cent kill. As pointed out by Pressman and Rhodes (1946) it is entirely academic whether by laboratory test germicides kill 100 per cent of the test organism or 99+ per cent, especially since by practical use tests such germicides have been proved effective disinfectants. The report by Quisno, Foter, and Rubenkoenig (1947) showed that a 1 to 30,000 dilution of a quaternary killed but 69 per cent of a total of 350,000,000 staphylococci in the test culture. It is important in this connection to note that in actual use such quaternaries are employed in 6 times this concentration, that is, 1 to 5,000 dilution. Also Kenner, Quisno, Foter, and Gibby (1946) have shown that 1 to 45,000 of a quaternary kills a sufficient number of *S. typhosa* to prevent infection as determined by intraperitoneal injection into mice of the medication mixture. The fact that sufficient numbers of the test organism were killed even by this high dilution, so that they failed to develop in the animal body, is further proof that less than complete kill by laboratory test is unimportant as a measure of germicidal effectiveness. As is clearly pointed out by Resuggan (1949), it is not necessary from a practical standpoint to require complete kill by laboratory test methods.

In a rebuttal of the papers by Klimek and Umbreit, and by Resuggan respectively, Klarmann and Wright (1948, 1949) stressed the following facts: Unlike the latter authors, Klimek and Umbreit worked with only one cationic compound, and they did not attempt to check the "semi-micro" procedure in every detail. No attempt has been reported by these authors to duplicate the highly relevant experiments with *Ps. aeruginosa*. Moreover, the phenomenon of massing of bacterial cells against glass surfaces was mentioned by other investigators in the recent past. It supplies the reason for the recommendation of a "swab method" made by Cade (1947a) which is found to parallel in efficiency the "semi-micro" technic of Klarmann and Wright. It was observed by Eckfeldt and James (1947) under certain conditions which, incidentally, gave rise to

the ingenious hypothesis that adsorption of the cationic substance by the glass reduced its bacteriostatic concentration in the immediate proximity of the glass below the inhibitory range, thus causing the *M. pyogenes* var. *aureus* colonies to develop adjacent to the glass. In another experiment, Eckfeldt and James showed that a bacteriostatic concentration in agar of a cationic compound nevertheless permitted the development of colonies of *M. pyogenes* var. *aureus* if the agar was spread upon a glass surface in a very thin layer. As to Resuggan's article the rebuttal revolves around the fact that this article is not based upon any original experimental work, but rather upon an inconclusive selection of quotations from the papers of different authors.

It is evident that the laboratory testing of quaternary ammonium compounds presents some difficulties. There is lack of agreement as to which procedures are most applicable, as well as how to interpret results obtained by the several methods suggested. One of the most serious factors involved is the apparent clumping of the test organisms in the medication tubes as a result of contact with the quaternary. McCulloch (1947) claims that false readings are obtained by the phenol coefficient method for the following reasons: the loop might not pick up a clump of agglomerated organisms (which tend to adhere to the surface of the tube); if picked up, such a clump might remain adherent to the loop when it is immersed in the broth subculture; and if the coated and agglomerated organisms were placed in broth, in which no additional particulate material was present, they might remain in a state of bacteriostasis and fail to give rise to growth. Also, in the case of plate counts in agar, many viable organisms in one clump would give rise to only one colony and hence would be read as only one viable organism. Since it appears that the tendency of organisms to agglomerate and adhere to surfaces following exposure to dilute solutions of the quaternary ammonium compounds has influenced the end points obtained with this method and the apparent velocity of disinfection when plate counts are made, the bactericidal efficiencies of these compounds should be re-evaluated.

McCulloch, Hauge and Migaki (1948a, 1948b) have reported results of laboratory tests on quaternaries in the presence of various kinds of organic matter met in veterinary practice. When exaggerated amounts of organic matter are employed in such tests the effectiveness of these germicides is materially reduced. While this is quite generally recognized, this limitation applies to many other germicides as well. This being the case, it is common practice, and actually specified by Federal and state health departments, that filth should be removed before disinfectants are applied. Where this

is done all disinfectants exert their full effect when used. It is for this reason, among others, that the presence of organic matter in laboratory methods of testing, and their interpretation and evaluation, are so important.

This is the purpose of the glass slide method suggested by Johns (1947). In this procedure the test organism is suspended in a 1 to 10 dilution of skim-milk applied to microscopic slides, drained, and partially dried. Slides are then immersed in the germicide, gently agitated, rinsed, placed in petri dishes, poured with agar medium, and incubated. Destruction of 99.9 per cent or more of the organisms is taken as the end point. This method approximates the conditions under which certain germicides, especially hypochlorites and quaternaries, are used in dairies, etc. The results of such tests can readily be interpreted in terms of practical value. Such "use-dilution" tests, as already stated, are most valuable for determining the germicidal effectiveness of quaternaries, hypochlorites, and other non-phenolic disinfectants.

A somewhat similar test procedure has been suggested by Goetchius and Botwright (1949) which is designed especially for testing sanitizing detergents recommended for use in cleaning and disinfecting milking machines and parts. Sterile rubber strips the size of ordinary microscope slides are immersed in a heavy suspension of the test organism in a 50 per cent water solution of sterile evaporated milk, drained, and dried for 10 minutes, then immersed in the quaternary disinfectant for 3 minutes, rinsed, and plated in Tryptone glucose agar containing 0.1 per cent Tamol as an inactivator, and incubated at 37°C for 48 hours. Controls with tap water are carried out in the same manner. Using *E. coli*, *S. fecalis*, and *Ps. aeruginosa* as test organisms, the sanitizing detergent must reduce the bacterial count 99 per cent or more below that of the control under the same conditions of test, in order to be considered satisfactory for use. A reduction of 99 per cent of all the test organisms may be expected to give entirely satisfactory results in practice, especially if the activity is that high against *Ps. aeruginosa*. Here again, the results of this test, a variation of the "use-dilution" method originally suggested by Mallmann and Hanes (1945), may be interpreted directly in terms of practical values.

Weber and Black (1948) found that results by the "use-dilution" technique conform to results obtained with suspensions of test organisms. After an extensive and detailed study of these various methods of test, they found that exposure of a water suspension of test organisms in glass medication tubes was eminently satisfactory for the purpose. They found that few, if any, of the test organisms adhered to the glass walls of the medication tubes and



that the use of glass tubes did not in any way affect the results of the test.

The procedure used by Weber and Black (1948) gives such constant results, with a minimum of skips and "wild pluses," that it may well become a standard method for the testing of quaternaries and other germicides used for food utensil sanitization. Of equal if not more importance is the fact that germicides passing this test have been found completely satisfactory for the disinfection of food utensils under practical conditions of use. This method conforms to the ideal for such a test in that it is based on practical use effectiveness, is technically sound as to every detail, and the results obtained are exact and easily duplicated. For these reasons the test should be seriously considered as a standard method for testing quaternaries.

The test organism, *E. coli*, is eminently satisfactory for this purpose. It is representative of the kinds of pathogenic bacteria present on food utensils, is more resistant than the non-sporing gram positive organisms which also may be present, is easily grown and identified, is safe for use in laboratory testing, is easily maintained at normal resistance, and is satisfactory as a test organism in other respects. All of these factors have been carefully considered by the proponents of the method.

A practical use test is recommended by the U. S. Public Health Service (1943) for regulating eating and drinking establishments. The method of test recommended includes plate counts from eating and drinking utensils obtained by means of swabs, the swabs being rinsed in water before plating. This is essentially practical testing and is not suggested as a laboratory procedure such as those discussed here. However, it is of interest to note that the requirements of this practical-use test are that the swab-rinse count must be less than 100 if the sanitizing operation is to be considered satisfactory.

A modification of this swab-rinse method has been proposed by a sub-committee on Research and Standards of the American Public Health Association (1944), for the control of food utensil sanitation. The area to be swabbed is the upper  $\frac{1}{2}$  in of the outer rim of cups and glasses and over a total area of approximately 4 sq in of forks, spoons, plates, etc. Standard tryptone glucose extract agar is used in plating the swab-rinse, the plates being incubated at 37°C for 48 hours. The average plate count per utensil surface must not exceed 100 if the sanitizing operation is to be considered satisfactory. Higher counts are indicative of inadequate bactericidal treatment. Here again, this is a practical-use test, not a laboratory method suitable for general testing and control of quaternaries.



Since at this time there is no standard laboratory method for determining the germicidal activity of quaternaries, these germicides are still being controlled by the phenol coefficient method. Although this method is not actually applicable to germicides that are not phenol-like, it has been found by experience that dilutions of quaternaries 20 times the phenol coefficient will be effective disinfectants when used in practice. In order to obtain results which are not fictitiously high due to bacteriostatic action in the subculture, the use of inhibitors is necessary (Quisno, Gibby, and Foter, 1946; Weber and Black, 1947; Lawrence, 1947; Weber and Black, 1948 and others). For this purpose duponal WA, lecithin, naphuride sodium, tergitol WA7, triton X 200, "Tamol," thioglycollate medium, etc., are employed. When such inhibitors are used, the phenol coefficient test, or modifications, may be employed for determining approximate dilutions for use in practice (Stuart, 1947).

While there is not as yet a standard laboratory method for the bacteriologic testing of quaternary ammonium compounds and other non-phenolic disinfectants, considerable progress has already been made toward such an acceptable procedure. It is evident that whatever method will finally be adopted will be based on practical-use conditions, or at least will reflect effectiveness under conditions of use. As already noted, this has been done in the testing of phenol-like disinfectants, and, as will be shown later, has been the basis of present methods of testing antiseptics.

A general review of surface action agents was prepared by Glassman (1948) in which a discussion of the bacteriostatic and bactericidal activity of such compounds was included. The purpose of this review was a general survey of the subject and was not concerned with the evaluation of methods of testing such compounds.

## ANTISEPTICS

Present methods of testing antiseptics are based on known values of clinical effectiveness. As emphasized by Reddish (1949) this is one of the most important factors to be considered in connection with laboratory methods of test.

Products classed as antiseptic may be either germicidal or bacteriostatic agents. Present laboratory methods of testing are designed to test for both kinds of antiseptic activity. In other words, our present methods of testing antiseptics are based on the definitions of antiseptics. They were developed by the U. S. Food and Drug Administration (Reddish, 1927*a*; Reddish, 1929; Circular 198, 1931) and have been generally employed for the testing and control of this class of drugs. Although other procedures have been sug-

gested from time to time, these methods are still widely employed for the purpose.

These methods will not be described in detail, but it may be of interest to examine the background of the various tests and to discuss their interpretation and evaluation. This subject is as important now as it was in the past, since the basis of the tests must be thoroughly understood before they may be properly interpreted. It was, of course, necessary to base these methods on known values of proved merit, since the procedures were intended to be used in the regulation and control of antiseptics in interstate commerce. For this purpose it was necessary to establish certain standards of comparison. The methods will be discussed separately in relation to the kind of antiseptics for which they were designed.

### LIQUID ANTISEPTICS

Since 2.0 per cent phenol had for many years proved effective as an antiseptic, the test for liquid antiseptics was designed to fit this germicide. Also, since antiseptics are used to prevent wound infections, it was logical to require them to kill the most common cause of such infections, *M. pyogenes* var. *aureus*. Then, as a factor of safety, the test was so designed that antiseptics were required to kill very large numbers of this infectious organism, far more than would be present in wounds, abrasions, cuts, etc.

This method of test makes use of a strain of *M. pyogenes* var. *aureus* (No. 209) which is representative of resistant staphylococci freshly isolated from suppurative infections (Reddish, 1925). The formula of the media employed was designed to maintain this resistance. The effect of peptone on the resistance of the test organism was emphasized and the particular brand used was specified. Details of the method of test were set forth (Reddish, 1927*a*, 1929), and the method became known as the U. S. Food and Drug Administration Method for testing liquid antiseptics (Circular 198, 1931).

The test for liquid antiseptics requires the use of approximately 350 million of the most resistant of all skin and mouth bacteria. This excessive number of staphylococci must be killed by liquid antiseptics within 5 minutes. It is evident that an adequate margin of safety has been provided.

Although 2 per cent phenol is generally recognized as a standard of comparison, antiseptics are not compared to phenol as in the phenol coefficient of disinfectants (Reddish, 1935, 1937). The test, therefore, classifies antiseptics as those that are at least equal to 2 per cent phenol and those that are not. In other words, products which pass this test may be considered antiseptic.

The general use of this test made it possible to standardize antiseptics on a sound basis (Reddish, 1928a). As a result, the control of antiseptics has been made more effective. Also, this effective control assures the professions and the public of antiseptics of known germicidal efficiency. This has repeatedly been confirmed in studies by laboratory and practical tests (Reddish, 1936) as well as by clinical experience in actual use of antiseptics.

Criticisms have been made of the method of testing liquid antiseptics because the test does not "simulate practical conditions of use." The test was never intended to duplicate clinical conditions for the simple reason that no single test can possibly simulate all the clinical conditions for which antiseptics are used. Since it is impossible to simulate all conditions, the best that can be done is to use a test that has been proved by actual clinical experience. This is exactly the situation in the case of this method, since the test is based on known values, as determined by practical and clinical experiences.

## PROPOSED NEW METHODS FOR TESTING LIQUID ANTISEPTICS

Within the past few years a number of new methods for studying and testing antiseptics have been proposed. Because of the above criticisms many *in vivo* tests have been suggested for the evaluation of the germicidal activity of antiseptics. Hunt (1937) made use of a practical test in which an "invasive" strain of *M. pyogenes* var. *aureus* was injected intracutaneously into mice which subsequently were injected with antiseptics at the same site after various time periods. It was found that once the necrotic and suppurative process had started none of the antiseptics injected later were effective in arresting the infection. However, in most instances infection was prevented by one of the antiseptics studied if injected within an hour following injection of the organisms. While this method of testing is useful in research on skin antiseptics, it does not lend itself to the testing of antiseptics for general use. Also, because of certain variables difficult to control, it is not applicable to the routine testing of even skin antiseptics and has not been widely used.

Another *in vivo* test designed specifically for the evaluation of skin antiseptics was developed by Nungester and Kempf (1942). This method, however, lends itself quite well to laboratory testing since the conditions of test can readily be controlled. For this reason this method is used in many laboratories throughout the country. The test is quite simple and the technique is easily mastered. How-





ever there are some features of the test that should be discussed in order to fully understand the usefulness and limitations of the method.

Test organisms are applied to the tip of the tail of white mice, after which the tail is dipped into the antiseptic, which is allowed to act for 2 minutes. The tip of the tail is then amputated and inserted into the peritoneal cavity of the same mouse. The results obtained are consistent and are not subject to uncontrollable variables. Much can be said in support of this test for the purpose of determining the value of antiseptics applied to the unbroken skin.

The principal objection to this test, however, is the nature of the test organisms used. In order that the test be effective, organisms pathogenic for mice (pneumococci and streptococci) must be employed. For this reason, results obtained with these test organisms cannot be interpreted in terms of practical value for human use. The pneumococcus is a very weak organism and is easily killed by antiseptics. A skin antiseptic which would pass this test by killing pneumococci would not necessarily kill the staphylococci which are normal inhabitants of the skin and which are the most common cause of wound infection.

The only other pathogenic organism used in this "infection prevention test" is a hemolytic streptococcus. This organism is an inhabitant of the skin and is a common cause of post-operative infection. Hemolytic streptococci, however, are much weaker than staphylococci, and therefore more easily killed by antiseptics. In fact, both pneumococci and hemolytic streptococci are only about half as resistant to germicides as *M. pyogenes* var. *aureus* (Reddish, 1927b). There is no assurance that the killing of hemolytic streptococci will mean that staphylococci will be similarly affected. There is, then, no margin of safety in this test, because weak test organisms are employed.

According to results obtained by the test, tincture of iodine 2 per cent was the only antiseptic that proved completely effective. While tincture of iodine is recognized as an effective skin antiseptic, satisfactory clinical results are obtained with a number of other antiseptics. Therefore, results obtained by this test are not fully supported by clinical experience.

Since this is the most reasonable *in vivo* test for skin antiseptics suggested so far, it is important to review the above objections to the test. As compared with other methods, this test is definitely weak because weaker test organisms are used. In addition, no provision is made for maintaining, or even assuring, the normal resistance of the test organisms. That is, there are no detailed specifications for the media employed for culturing the test organisms.



It would therefore be difficult to duplicate results with different strains of these test organisms, or even with the same strains in different laboratories. There is no precise standardization of the procedure. It must be borne in mind also that use of this test is limited to skin antiseptics and is not intended as a test for general antiseptics.

If this test were perfected to include the above specifications, and if a factor of safety were included, the method might well be adopted as a standard procedure for the laboratory testing and control of antiseptics specifically recommended for disinfection of the skin.

Price (1938, 1939) developed a very satisfactory method for determining reduction in numbers of bacteria on the skin. While the test was designed originally for determining the effectiveness of soap for the removal of bacteria from the skin in surgical scrub-up, it is applicable also for evaluating the effectiveness of germicides recommended for the same purpose. Actually, it is the best and most accurate method available for this purpose. The Cade (1950) modification of the test is now widely used.

Although the method is time-consuming, it is simple and may be easily employed in any laboratory. The technique follows closely the pre-operative scrub-up procedure employed in hospitals. Plate counts are made of water rinses before and at specified time periods after the use of soap or detergent, medicated or non-medicated, or alcohol and other germicides employed after the scrub-up procedure. Care must be exercised and special precautions taken to avoid bacteriostatic action when certain germicides are used. The method as described originally does not include such precautions since it was intended only for testing soaps and such low bacteriostatic germicides as alcohol. This is entirely a practical test in which only the normal skin flora is involved.

## TOXICITY TESTS

There have been many suggestions that the laboratory testing of antiseptics should not only simulate practical conditions of use but should, if possible, also indicate the degree of toxicity of antiseptics for human and animal tissue. Efforts have been made to devise tests combining these two features. Some of the methods suggested merely test for germicidal activity and toxicity to certain specialized tissue cells.

Among the first such methods presented was one which made use of a "toxicity index," obtained by exposing both embryonic chicken heart tissue and *M. pyogenes* var. *aureus* to antiseptics

in order to compare inhibition of tissue growth with germicidal action (Salle, McOmie, and Schechmeister, 1937). While this is an interesting method, its use obviously is limited to research. It is an *in vitro* method that does not simulate practical use conditions of antiseptics and evidently was not intended for this purpose.

The highest dilution of the germicide preventing growth of embryonic chicken heart tissue during incubation for 48 hours *in vitro* is compared to the highest dilution of the germicide killing *M. pyogenes* var. *aureus* within 10 minutes but not in 5 minutes. The result is designated as a "toxicity index." A "toxicity index" of less than 1.0 means that the germicide is more toxic against bacteria than against embryonic chicken heart tissue and, if the index is more than 1.0, the reverse is indicated. Although the result is designated as a "toxicity index," it is apparent that the nature of the test does not justify its interpretation in terms of practical values.

In the first place, no relationship exists between embryonic chicken heart muscle and skin, mucous membrane, or areas surrounding wounds to which antiseptics are usually applied. Toxicity to embryonic chicken heart muscle does not mean equivalent toxicity to epithelial tissue, for example. Therefore the test is not a practical toxicity test.

Furthermore, results obtained by this "toxicity index" test do not properly classify antiseptics in terms of usefulness under clinical conditions. For example, it was necessary to use a 1 to 1,800 dilution of iodine in order to arrive at a "toxicity index" for this antiseptic. Actually there is no clinical relationship between the toxicity to tissue of a 1 to 1,800 dilution of iodine for clinical use, and the standard, official tincture of iodine, either the mild (2 per cent) or the strong (7 per cent). Therefore, no practical interest could pertain to the lack of toxicity of aqueous iodine of dilutions greater than 1 to 1,800 when concentrations of 2 and 7 per cent in alcohol are the ones used in practice.

Since the term "toxicity index" as used in this connection includes a consideration of both germicidal activity and toxicity to tissue, the "toxicity index" figures do not actually reflect tissue toxicity alone. However, the general use of the term may and often does indicate only toxicity as applied to tissue. In fact, this is the usual connotation of the term and its general use as reflecting the results obtained will often be misleading.

For example, under the conditions of this test it has been shown that Mercurochrome has a "toxicity index" 2,620 times greater than iodine. Yet it cannot be shown that 2 per cent Mercurochrome is 2,620 times more toxic to human tissue than 2 per

cent tincture of iodine. As a matter of fact, if Mercurochrome actually were 2,620 times more toxic than iodine, it would not be safe to use. Actually by this test it is only 6 times more toxic to chicken heart muscle tissue ( $10,500 \div 1,800 = 6$ ) and not 2,620 as the index indicates. Because results show that the "toxicity index" of Mercurochrome is 262 and that of iodine is only 0.09, such an "index" is misleading.

To recapitulate, the following are valid objections to this "toxicity index" test procedure: (1) embryonic chicken heart muscle does not represent human or animal skin, mucous membrane, or tissue exposed in wounds; (2) the test requires exposure of the tissue to antiseptic for 48 hours, whereas in actual use antiseptics are ordinarily applied for much shorter time periods—in some instances only minutes; (3) concentrations of the antiseptics used in this test are many times weaker than the concentrations used in practice; and (4) conditions of the test do not simulate the clinical conditions under which antiseptics are used. Therefore, the results obtained by this test cannot be interpreted in terms of practical value.

Another suggested method of evaluating antiseptics by comparison of germicidal activity with tissue toxicity is one in which leukocytes and skin are employed as tissue (Nye, 1937). This method, published at about the same time as the above "toxicity index" method, has many desirable features and approximates clinical conditions more closely than many other laboratory test procedures. This method is a combination of (1) germicidal tests for liquid antiseptics, (2) diffusibility tests through paralodion sacs, (3) a toxicity test against leukocytes, and (4) tests for intradermal toxicity.

The object of this test is to determine which antiseptics are best suited for use in wounds, cuts, abrasions, and cavities—in other words, for surgical use. The ideal antiseptics for surgical use should possess positive germicidal activity against pus-forming organisms, should be active in the presence of serum and blood, should be diffusible (penetrate into body tissue), should be relatively non-toxic in the dilutions employed, and should be inexpensive. The author of the test has considered all of these factors in his study.

In this test, the *in vitro* method for liquid antiseptics is used, both with and without blood serum, for determining germicidal activity. Diffusibility tests are made with paralodion sacs, the antiseptics being placed inside the sacs and the dialysate tested for germicidal activity. The test for toxicity makes use of white blood cells (leukocytes) which constitute an important defensive factor



against infection. When they are killed by a germicide, they are prevented from accomplishing their normal function of engulfing and killing infectious bacteria. The test is designed to show whether or not white blood cells are killed by germicides in the dilutions usually employed. The tests for intradermal toxicity were conducted by injecting dilutions of the solutions into the abdominal skin of white rabbits and observed for 6 days; the lowest dilution producing no necrosis was considered the nontoxic intradermal dose.

In some respects this test is superior to the first "toxicity index" test just described. In the first place, leukocytes and human skin are employed instead of embryonic chicken heart tissue. Also, exposure times to the tissue and to the test bacteria (*M. pyogenes* var. *aureus*) are comparable, not widely different as in the above "toxicity index" test, in which tissue is exposed to test antiseptics for 48 hours and to *M. pyogenes* var. *aureus* for only 10 minutes. The tests for penetration through blood serum and the tests for diffusibility through paralodion are of much importance, and results obtained are of significance in terms of practical value.

The only serious objection to the method is the use of leukocytes as one of the media of test for tissue toxicity. Leukocytes do not represent body tissue generally and the effect of antiseptics on leukocytes cannot be interpreted as effect on those human and animal tissues to which antiseptics are ordinarily applied. Also, leukocytes are single cells that may be easily surrounded by liquid antiseptics, whereas cells of other tissues, such as skin, mucous membrane, and muscle, are not so easily attacked by liquids. Certainly no practical "toxicity index" or "toxicity test" should be based only upon effect on leukocytes.

Because leukocytes are a part of the body's defense mechanism, it has been suggested that they should not be destroyed by an antiseptic, since by doing so this defense factor would be eliminated. However, if leukocytes are destroyed in a wound, more are supplied to the site of injury, for there is practically an inexhaustible supply of leukocytes in the body. Therefore, it is of no practical importance if those present in the area are destroyed at any one time, since more are continually supplied to take their place. It is far more important to kill the invading infectious bacteria, even if those leukocytes present locally at the time are destroyed.

A few years later a second method for computing a "toxicity index" by the use of leukocytes was suggested (Welch and Hunter, 1940). This test will be discussed in some detail because, according to the authors, "In the development of the proposed method an attempt has been made to approach as nearly as possible the conditions under which antiseptics are used." The method proposed resembles

the one just discussed, in that leukocytes are used as tissue for testing toxicity. But the test fails to meet the objective of simulating actual conditions under which antiseptics are used. In fact, it does not approach this ideal as closely as the last described method (Nye, 1937) since it makes no provision for testing for toxicity against such tissue as skin nor does it test for diffusibility and penetration of tissue by antiseptics.

Under the conditions of this test, the leukocytes and the test organism *M. pyogenes* var. *aureus* are exposed to the antiseptic *in vitro* for 30 minutes. Observations are made as to the ability of the leukocytes to phagocytize the staphylococci. The "toxicity index" is determined by dividing the highest dilution of the antiseptic preventing phagocytosis, that is, "toxic" for the leukocytes, by the highest dilution killing the test organism. If presence of the antiseptic inhibits phagocytosis in a dilution greater than the germicidal dilution, the "toxicity index" is above 1. If the dilution inhibiting the leukocytes is more concentrated than that killing the test organism, the "toxicity index" is less than 1. It is of interest to note that inhibition of phagocytosis is designated as "toxicity to tissue" and not simply as inhibition of leukocytic activity. Also, the figure obtained by dividing the dilution inhibiting phagocytosis by the dilution killing *M. pyogenes* var. *aureus*, is designated as the "toxicity index."

As explained earlier, although leukocytes are tissue, leukocytes do *not* represent those body tissues to which antiseptics are ordinarily applied. They do not possess resistance to antiseptics and other drugs comparable to such tissue as epithelium, mucous membrane, etc. Therefore, inhibition of leukocytes cannot be considered as general "tissue toxicity."

The results first reported by this test do not show whether or not the leukocytes themselves were actually destroyed or even injured. All that is shown is that phagocytosis was prevented by the antiseptic. It was not shown why the leukocytes were rendered inactive. Since leukocytes can be restrained from exercising their phagocytic function without a toxic effect being exerted—for example, by interference with humoral elements in the blood, such as opsonins—the results of this test can not be properly designated as a "toxicity index."

In another paper (Welch, 1939) it has been shown that inhibition of leukocytes by antiseptics is due to destruction of opsonic complement, or opsonins. Since opsonins, normally present in the blood, are necessary in order for leukocytes to kill bacteria, anything which destroys this substance will prevent phagocytosis. Opsonins are non-living, therefore their destruction is not due to toxic action.

If chemical alteration (destruction) of opsonins is the reason for inhibition of phagocytosis in this test, the results obtained could not possibly indicate "toxicity" of the antiseptics.

This test has been applied to oral antiseptics (Welch and Brewer, 1942), despite the fact that neither opsonins nor leukocytes, in any quantity, are present in the mouth and throat. Opsonins are normally present in blood only. Since blood is not present in the normal mouth, any leukocytes which may possibly be present in the oral cavity cannot phagocytize bacteria for the simple reason that activating opsonins are not present. This fact must have been recognized by the authors of the method because in testing oral antiseptics they found it necessary to add normal serum to a mixture of leukocytes in saliva in order to produce phagocytosis.

This "toxicity index" lacks value even in testing antiseptics recommended for use in cuts and wounds, for reason that leukocytes act only in tissue. If bacteria and leukocytes are killed on the surface of a cut or wound, no harm will result, since active leukocytes present in the capillaries will continue to function. Also, new phagocytes and opsonic complement are continuously carried to the site of injury. Actually thousands of times more leukocytes and opsonic complement are removed when blood is taken for a transfusion than could possibly be destroyed by the local application of an antiseptic to a cut or wound, yet no harm results.

The authors of this test report that iodine is the only antiseptic showing a "toxicity index" of less than 1, that is, iodine is the only antiseptic that is less toxic to leukocytes than it is to *M. pyogenes* var. *aureus*. However, in order to be able to assign a "toxicity index" of less than 1 to iodine, it was necessary to dilute the official iodine solutions 1 to 60 and 1 to 84. There is no clinical evidence, however, that 1 to 60 Lugol's solution and 1 to 84 dilution of 2 per cent tincture of iodine are germicidally effective under conditions of use. The only clinical evidence of the value of iodine solutions as antiseptics is the use of undiluted Lugol's and tincture of iodine.

For all of the reasons presented, it becomes apparent that the "toxicity index," as determined against leukocytes, is without practical significance. The indices can be of only theoretic interest. The test itself should be reserved for use only in chemo-therapeutic research. It should not be employed as a test for the practical evaluation of antiseptics.

In a still later paper (Welch, Slocum, and Hunter, 1942) this test was modified to show actual destruction of leukocytes, instead of mere inhibition. The results are reported as "toxic activity," using human blood as the test tissue. The irreversible loss of phagocytic action after 10-minute exposure to antiseptic is taken as an



indication of cell death, and the results obtained are designated as "toxicity of antiseptics" despite the fact that leukocytes are not representative of body cells generally.

It is interesting to note that the two methods using embryonic chicken heart muscle and leukocytes give results which are somewhat comparable. That is, germicides shown to have a low "toxicity index" by one method give a low figure by the other method, while others give progressively higher indices by both methods. This would seem to indicate that both methods are satisfactory for the purpose. From a strictly technical point of view this is a logical conclusion, but as just stated, neither of the methods can be interpreted in terms of practical use value.

On the other hand, the results determined by these two methods are not in accord with results obtained by other toxicity studies on germicides. For example, Lambert and Meyer (1926) used rabbit spleen as tissue in a comparative study of the toxicity of certain germicides to tissue and staphylococci. Using an exposure time of 20 minutes, they found that iodine and mercuric chloride gave more favorable results by this method than some of the newer antiseptics. Although no toxicity indices were calculated from the figures obtained, the results showed iodine to be equally toxic to splenic tissue and staphylococci; that 33 per cent alcohol killed tissue cells and 50 per cent alcohol killed staphylococci; 1 to 2,500 mercuric chloride killed tissue cells and 1 to 10,000 killed staphylococci; 1 to 500 Mercurochrome killed tissue cells and 1 to 250 killed staphylococci; et cetera. If toxicity indices were calculated as in the two methods discussed above, the results obtained would be quite different from those reported by Salle *et al.*, and Welch *et al.* It would seem from this that the "toxicity index" obtained would depend upon the tissue used and the method employed.

This is further demonstrated by Bucksbaum and Bloom (1931) who used cultures of spindle cells and periosteum from 12 day embryonic chicks as tissue, and *M. pyogenes* var. *aureus* as the test bacterial culture. The time periods of exposure were 24 and 48 hours. When the greatest dilution required to kill the staphylococci was compared to the greatest concentration in which the tissue cells showed normal growth, the following indices were obtained: phenol, 0.2; iodine, 0.5; Mercurochrome, 0.5; Metaphen, 0.6; and Merthiolate, 0.9. Here again these "toxicity indices" are quite different from those reported by other investigators and offer further proof that results from toxicity studies on germicides compared to germicidal activity will vary according to the procedures used.

Another method for determining "toxicity indices" of antiseptics was suggested by Bronfenbrenner, Hershey, and Doubly (1938,

1939). The method used was intended to simulate as nearly as possible conditions met in the animal body, since the method employed by others did not seem to fulfill this requirement, and also because other techniques were time-consuming and laborious. This method proved simple, rapid, and quite flexible. The method is a comparison of the effects of germicides on liver cells of the adult mouse and on suspensions of *E. coli* and *M. pyogenes* var. *aureus*. Depressant effect on the rate of oxygen consumption as determined by the manometric method was taken as a measure of destructive action in either case. When results of these tests are calculated in terms of "toxicity indices" it is shown, for example, that phenol has an index of 1.1, iodine (tincture) 2.6, and mercuric chloride 0.21. It is further shown that the tissue toxicity results obtained by this method are in close agreement with values obtained by intraperitoneal injection into mice. However, the results obtained by this method are quite different from those reported by Lambert and Meyer; Bucksbaum and Bloom; Salle *et al.*; Welch *et al.*; and others. While it would be difficult to choose between these various methods, it is apparent that the manometric method more nearly simulates conditions met with in the body than do the other procedures suggested. As specifically stated by the authors, this method is useful for preliminary evaluation of new germicides, and in studies on their potential practical value under clinical conditions of use. It must be emphasized again that actual use under clinical conditions is the final criterion as to the toxicity of antiseptics and their safety in specific application.

For example, there is wide divergence in the "toxicity index" of iodine and Mercurochrome as reported by various investigators. It is of interest to note that under practical conditions of use no such differences are experienced. Reddish and Drake (1928) and Smith (1932) in comparative studies of tincture of iodine and alcohol-acetone-aqueous solutions of Mercurochrome gave equivalent results when used for the disinfection of skin, with no apparent difference in toxic effect. Other examples of safety with clinical effectiveness of germicides having high "toxicity indices" have also been reported.

The same situation exists, of course, in the testing of antiseptics generally. This is emphasized by Spaulding and Bondi (1947) with respect to the use of tissue toxicity tests in connection with any study of the infection-prevention of antiseptics. As indicated previously, the infection-prevention method of testing skin antiseptics of Nungester and Kempf (1942) is the most suitable such test proposed so far, in spite of certain deficiencies which have been discussed. However, this method does not provide for testing toxicity.

Spaulding and Bondi have provided such a toxicity test, in connection with the infection-prevention method of Nungester and Kempf. This combined method offers so much promise as a possible standard procedure that further analysis and evaluation is justified.

In the first place, the test is simple and does not require special techniques nor special equipment. It can be performed in any bacteriological laboratory. The results obtained are definite and readily reproducible. Further, the results obtained can, with reasonable caution, be interpreted in terms of practical value. Also, proper evaluation of skin antiseptics can be assured only by such a comparison of infection-prevention and toxicity.

Spaulding and Bondi, using Nungester-Kempf procedure, designated the highest dilution of the antiseptic preventing infection in one-half of the mice as the infection prevention index (IP/50). Using the same technique, but eliminating use of the test organisms, the greatest concentration of the antiseptic which fails to kill one-half the mice is designated as the toxicity index (T/50). Since the same *in vivo* technique is used in both tests, direct comparison of the two is justified. Then IP/50 divided by T/50 can be designated as the infection prevention-toxicity index, which indicates the difference between bactericidal activity and toxicity. Consequently, the higher the IP-T index value of the skin antiseptic, the more satisfactory it is likely to prove for practical use under clinical conditions.

Spaulding and Bondi used as the test organism a type I pneumococcus of such virulence that 5 to 13 were lethal for mice by intraperitoneal injection. Using this organism tyrothricin gave an IP-T index of 712,000 and Compound G4 an index of 430, whereas Compound G214 gave an index of 2, Zephiran 4, Iodine 4, and Metaphen 3. Although these compounds are used as skin antiseptics, they are also widely used for application to cuts and wounds with consequent opportunity for absorption. For this reason, and also since there is as yet no practical and reproducible method for evaluating skin toxicity, the IP-T index may well be used for evaluating antiseptics generally. In support of this method it must be emphasized that infection-prevention and toxicity are obtained by the same technique and that these are *in vivo* tests. Also, it has been shown that results obtained by this method are comparable to those obtained by the phagocyte-toxicity method of Welch and Hunter, as well as the LD/50 intraperitoneal method. The toxicity relationships, as shown by Spaulding and Bondi, are in general agreement by these three methods.

The principal objection to this method, as previously noted for the infection-prevention test of Nungester and Kempf, is use of the



pneumococcus as the test organism. This is not a skin organism, nor is it a factor in wound infections. Furthermore, it is much less resistant to germicides than skin bacteria, especially the staphylococci. Realizing this, Spaulding and Bondi attempted to use the Smith strain of a staphylococcus in a series of infection prevention-toxicity tests. These tests were not satisfactory, since survival rates were inconsistent and not reproducible. They suggested the possibility of using the mouse-virulent Smith strain in such tests, provided cultures were swabbed on the tail, mucin introduced into the abdominal cavity, and test mice cultured for staphylococci after 2 to 3 days, without waiting for death to occur. It is evident that this organism should be used if possible, since it is the most common skin organism and the most common cause of infection in cuts, wounds, and skin injuries generally.

Similar attempts were made by Spaulding and Bondi to use a streptococcus, with results that were in general intermediate between those obtained with the pneumococcus and staphylococcus. Here again the streptococcus is less resistant to germicides than the staphylococcus and should not be used in testing antiseptics if it is possible to use a staphylococcus. In spite of these objections and the lack of certain detailed specifications, the infection prevention-toxicity method of Spaulding and Bondi appears to be the best *in vivo* method that has so far been suggested for accurately evaluating antiseptics.

In this connection, reference may be made to the so-called self-disinfecting power of the skin as reported by Arnold, Gustafson, Hull, Montgomery, and Singer (1930). This fallacy was disproved by Norton and Novy (1931, 1932), Price (1938), and others. Needless to say, it is unwise to depend upon so-called self-disinfection of the skin, for reasons demonstrated in clinical practice and proved over the past few years.

### IN VIVO TESTS

Still other *in vivo* tests have been suggested, some of which are definite improvements over previous methods. One such *in vivo* test, which improved previous methods, makes use of the skin of white mice and *S. pyogenes*, a test organism that is capable of causing peritonitis (Sarber, 1942). The streptococcus culture is first applied to the slightly abraded skin of white mice and the germicide is then applied to the area for a specified time period. Pieces of the treated skin are then inserted into the peritoneal cavity of the mouse. The germicide is allowed to act 3 minutes before bits of skin are inserted into the peritoneal cavity. Failure to develop infection is proof that the test organism was killed by the antiseptic.

By this test, tincture of iodine gives the best protection against infection. Other antiseptics tested are much less effective, in varying degrees. The only objection to this method is that the test organism, *S. pyogenes*, is much more easily killed by germicides than is *M. pyogenes* var. *aureus*, the most common inhabitant of the skin. So for this reason the test does not provide the important factor of safety.

Another of the new *in vivo* methods for testing activity of antiseptics uses as tissue the chorio-allantoic membranes of eggs containing live embryos, and *M. pyogenes* var. *aureus* as the test organism (Green and Birkeland, 1944). The chorio-allantoic membranes of eggs containing embryos 12 days old are first inoculated with a small quantity (0.02 ml) of a dilute (1 to 10) suspension of broth culture of *M. pyogenes* var. *aureus*. Eighteen hours later and on each of the 5 following days, 0.2 ml of the test antiseptic is dropped on the membrane. The day after the last treatment, the membrane is stroked with a cotton swab, then streaked on an agar plate. After incubation, the plates are counted for numbers of surviving organisms.

By this method penicillin and the cationic detergents tested were found to be effective in killing the test organism, whereas iodine, phenol, and the organic mercurials employed were ineffective. It is inferred from these results that penicillin and the cationic detergents should possess therapeutic value under clinical conditions of use, whereas iodine, phenol, and the organic mercurials would not be clinically effective.

While this method of test simulates wound conditions to some degree, it still fails to satisfy the conditions required of a satisfactory test procedure for antiseptics. The chorio-allantoic membrane of a fertile egg does not simulate such human or animal tissues as skin or mucous membrane. Also, the amount of culture used in the test is too small to allow for a factor of safety. But still more important, the antiseptics tested were applied in far greater dilution than is recommended for use in clinical practice. For example, iodine was used in dilutions of 1 to 2,500, 1 to 5,000, and 1 to 10,000, whereas 2 and 7 per cent iodine are used in clinical practice, that is, a 1 to 50 solution of iodine is the weakest dilution acceptable for general use. And finally, there is no provision in the method for preventing bacteriostatic activity of the test antiseptics when agar plates are swabbed from the treated membranes.

It is interesting to note that the antiseptics giving the best results by this test are those that are highly bacteriostatic against the test organism, *M. pyogenes* var. *aureus*. It could well be that their bacteriostatic effect on the agar plates accounts for the favor-

able results reported. On the other hand, iodine, phenol, and Azochloramide, in the dilutions used in the test, are not so highly bacteriostatic under the conditions of this test, therefore it would be expected that unfavorable results be obtained from the dilutions used. Also, Metaphen and Merthiolate could hardly be expected to exert their full effect in the dilutions used, namely, 1 to 5,000 to 1 to 30,000, when they are actually recommended for clinical use in 1 to 500 to 1 to 1,000 dilutions.

Here again it is necessary to take a practical view of the problem. While it may be desirable to use a dilution of antiseptic that would not injure such delicate tissue as the chorio-allantoic membrane of fertile eggs, in human and animal practice it is far more important to kill the infective organisms. Infection must be controlled even if the antiseptic does destroy some of the tissue cells. In actual practice, antiseptics are used in concentrations that kill infective organisms, such as 1 to 50 phenol, 1 to 50 iodine, 1 to 500 Metaphen, 1 to 1,000 Merthiolate, etc. Even though these concentrations of the antiseptics do destroy chorio-allantoic membrane, still they prevent infection by killing the invading bacteria, and at the same time they cause no serious damage to the human or animal tissues on which they are used.

## PROFILE EVALUATION OF ANTISEPTICS

It is, of course, desirable and even necessary to conduct a variety of tests on antiseptics in order to evaluate their possibilities for clinical use. This is the general consensus of bacteriologists and physicians actively interested in this field. Therefore tests should be conducted to determine their various properties under a variety of conditions. One single test is not sufficient for this purpose and, in fact, it is not expected that any one test will supply all of the information necessary to evaluate the properties of an antiseptic.

Recently a very excellent suggestion has been made for such a profile evaluation of germicides (Salle and Catlin, 1947). A combination of several well-established tests is recommended for the purpose. Use of these tests is specified for determining the following properties of antiseptics: (1) the highest dilution of germicide capable of killing *M. pyogenes* var. *aureus*; (2) extent of bacteriostasis; (3) influence of organic matter; (4) speed of action; (5) penetrability; and (6) toxicity.

For this purpose the following tests are employed: (1) modification of the *in vitro* test for determining killing dilution against *M. pyogenes* var. *aureus* (using standardized suspension); (2) bac-



teriostatic tests in 10 ml and 100 ml broth and thioglycollate medium; (3) similar tests in the presence of 10 per cent horse serum; (4) tests for rapid germicidal activity, in time periods from 1 to 10 minutes; (5) penetration tests, using the Agar Cup Plate Method, both with and without 10 per cent horse serum; and (6) toxicity tests on embryonic chicken heart tissue.

Studies of this character give considerable information relative to the properties of antiseptics. In fact, a combination of these tests will give information from which the practical value of an antiseptic may be predicted, or at least they will reflect its potentialities. Furthermore, the methods employed are all well-known procedures that have been used separately for many years. It is now proposed that all of these tests be conducted as a panel. Since each test shows certain specific properties of antiseptics, collective results give a correct and useful profile of the antiseptics tested.

In addition to using a variety of tests to evaluate the potential value of antiseptics and other germicides, it is also necessary to conduct tests on different representative pathogenic organisms. Reddish (1925), Philbrick (1929), and others have recommended that such studies be made according to the nature of the germicide being investigated as well as the specifications as to its use.

Recently a serious effort has been made to determine such a germicidal spectrum of various kinds of antiseptics against a variety of test organisms (Ostrolenk and Brewer, 1949). The method employed is a variation of the phenol coefficient type of test procedure, using a large number of representative pathogenic organisms. The purpose of the study was to determine the germicidal activity of representative germicides, including mercury compounds, phenol, cresol, hydrogen peroxide, alkali, acid and alcohol, silver nitrate, iodine, and quaternary ammonium compound. Fourteen microorganisms, of pathogenic or potentially pathogenic species, were used as test organisms. Cultures were grown in 2 per cent trypticase and after exposure to various dilutions of antiseptics were subcultured into U.S.P. liquid thioglycollate medium.

Results of these tests showed the wide variation in resistance of different species of the same test organisms and also between the various classes of bacteria. It is evident from results obtained that the resistance of test organisms must be determined before they are employed in laboratory tests on germicides. Also, it was shown that it is desirable to use a variety of such test cultures in order to obtain a full bactericidal spectrum. This test should, by all means, be included in studies of the germicidal activity of antiseptics.

## MERCURIALS

During the past few years much interest has been shown in the laboratory testing of mercurial germicides, especially the organic mercurials. The matter of proper procedure for testing these compounds and the interpretation of results of such tests have been open to question. Shippen (1928) and others have shown the fallacy of using standard methods for this purpose. Since the efficacy of a whole class of germicides has been brought into question, a discussion of recent developments in this field is necessary.

One of the principal difficulties in laboratory testing of mercurial germicides has been the high bacteriostatic activity of such compounds (Shippen, 1928; Brewer, 1939). The difficulty has largely been overcome by use of thioglycollate as the subculture medium (Brewer, 1940). Use of this medium in the testing of mercurial antiseptics permits surviving organisms to grow. Thus the true germicidal properties of mercurials are determined and much of the confusion caused by bacteriostatic activity in the subculture media is eliminated.

It has been generally known for many years that mercurial antiseptics are more highly bacteriostatic than germicidal. This has been proved repeatedly by laboratory and practical tests, both *in vitro* and *in vivo*. Therefore, the information given in recent publications on the subject is not surprising. Interpretation of the new data presented, however, must be considered in the light of present knowledge of the whole matter of testing antiseptics and also in terms of practical application.

Before discussing controversial aspects of the laboratory testing of organic mercurials, it is important to keep in mind the definition of the word "antiseptic," for the accepted definition of the term covers both bactericidal and bacteriostatic properties. It is immaterial which of these properties an antiseptic possesses, provided it renders infectious organisms innocuous when used as directed—that is, if it acts "against infection" under clinical conditions of use.

Recently a thorough restudy of the antiseptic properties of certain organic mercurials has been made (Morton, North, and Engley, 1948). The objectives of the study were to determine "(1) the effect of some of the organic mercurial compounds on an organism pathogenic for man and mouse and (2) whether the growth of the test organism in thioglycollate parallels infectivity for the animal body."

The organism used in this study was *S. pyogenes* (Strain C 203 M) which gives a wide zone of hemolysis on blood agar and

is pathogenic for man and some laboratory animals. White mice were regularly killed with this organism by intraperitoneal injection of 1 ml of a 1 : 10,000,000 dilution of a 24-hour culture.\* This lethal dose gave only four colonies when grown in blood extract agar.

The organic mercurials employed in the studies were Mercurochrome, Merthiolate, and Metaphen. The technique employed in the test procedure differed in some essential features from present standard methods. The medication temperature was 20°C instead of the standard 37°C temperature used for testing of antiseptics. Subcultures were made by inoculating 0.5 ml of the antiseptic-culture mixture into subculture media, instead of 1 loopful as in the standard procedure. These two modifications alone render this test much more severe than the already severe test for liquid antiseptics. Also, the number of lethal doses employed, namely 5,000,000, is much higher than is required for simulating practical conditions of use. In other words, the test requires the killing of 5,000,000 lethal doses of *S. pyogenes* at 20°C. This is loading the test against the antiseptics far beyond the point necessary to test practical values.

The results of these tests, as might be expected, appear to demonstrate that the organic mercurials employed are weak germicides. These results, however, cannot be interpreted in terms of practical value for the simple reason that the conditions of the laboratory test are too severe.

The conclusions the authors draw from their results are untenable as a clinical evaluation of these mercurial antiseptics. This is clearly emphasized in criticisms of the paper published later (Brewer, 1948; Powell, 1948). It was shown that when the number of test organisms is reduced to that approaching practical clinical conditions, both Mercurochrome and Merthiolate are demonstrated to be actively antibacterial in effect—even under the conditions of a test procedure not suitable for testing antiseptics, that is, at 20°C medication temperature. In fact, more than 80 per cent of the test animals survived excessive lethal doses of test organisms that had been exposed to the antiseptics at 20°C for 10 minutes. When the intravenous method of inoculating the animals was used instead of the intraperitoneal route, none of the animals were killed by massive lethal doses exposed to the antiseptics. Also, these results were obtained with a concentration of antiseptic only one-half that marketed to the professions and the laity. Even when 100,000 lethal doses of the streptococci were used, these antiseptics proved germicidal under the conditions of this test method. These re-evaluations of the original tests prove that effectiveness of the



mercurial antiseptics tested depends upon time, temperature, and number of organisms used.

In this connection, it is well to emphasize the fact that when used clinically, antiseptics continue to act for varying periods of time. In the case of organic mercurials, this is usually an extended period, certainly far longer than the time periods used in these tests. Some of these germicides act slowly but, since they are also bacteriostatic during the period of exposure, this is no real disadvantage.

On the other hand, some antiseptics kill bacteria quickly, but are not highly bacteriostatic. *Liquor Antisepticus N. F.* and similar germicides belong to this class. In fact, one such antiseptic has been shown to kill hemolytic streptococci within 15 seconds when 0.5, or even 1.0 ml of undiluted broth culture of these organisms is exposed to 5.0 ml of the antiseptic at 37°C. Such quick-acting germicides, however, are not necessarily better than the slower-acting organic mercurials. They act "against infection" in their own way. Both are effectively antiseptic under clinical conditions of use.

Of course, the final criteria for an antiseptic are effectiveness and usefulness in clinical practice. The vast amount of published data supporting the clinical effectiveness of organic mercurials constitutes sufficient evidence of their value as antiseptics for general use. This extremely important factor should be given first consideration in any study of antiseptic properties, not only of organic mercurials, but of all antiseptics.

## ANTISEPTIC OINTMENTS

The method for testing liquid antiseptics described earlier is employed for testing preparations applied clinically for short time periods such as those used in first aid, preoperative skin preparation, the oral cavity, etc. Antiseptic ointments, on the other hand, are tested by an entirely different method, since customarily they are applied for long periods of time. Because such antiseptics are in continuous contact with the bacteria present, they need only be bacteriostatic in order to prove effective in rendering infectious organisms innocuous.

Therefore, the laboratory test for antiseptic ointments is quite different from the test for liquid antiseptics. The method usually employed is known as the Serum-Agar Plate Method (Reddish, 1929).

The size of the zone of inhibition given by this method has a bearing on the clinical effectiveness of the ointment being tested.

Since Ammoniated Mercury Ointment, U.S.P., has been widely and successfully used as an antiseptic ointment for a great many years, it is used as the standard of comparison in the test for antiseptic ointments. A clear zone of inhibition of at least 0.2 cm in width and 0.2 cm in depth is given by this standard antiseptic ointment. Antiseptic ointments tested by this method must, therefore, give a minimum zone of 0.2 cm if they are to be considered effective. Most ointments give a clear zone of inhibition greater than 0.2 cm. This test simulates actual conditions of use to some degree, since the semisolid serum-agar is somewhat comparable to wound exudate and tissue (Reddish, 1947*a*, *b*).

#### ANTISEPTIC WET DRESSINGS, DYES, POWDERS, ETC.

When liquid antiseptics used as wet dressings, as well as antiseptic dyes, powders, etc., are employed clinically, they remain in contact with bacteria for long periods of time. Therefore, the method of test is designed to determine bacteriostatic activity, not germicidal effect as in liquid antiseptics for general use. The test procedure is similar to that for antiseptic ointments, except that the preparation being tested is applied in a 1.5 cm cup made in the agar. Antiseptic powders are applied in the same manner.

Interpretation of the results obtained with these preparations is comparable to that given above for antiseptic ointments, except that the acceptable zone of inhibition is somewhat larger. An antiseptic dye solution of known merit, merbromin (Mercurochrome), which has been widely and successfully used in surgery and first aid, gives a zone of 0.7 to 0.9 cm by the method described above (Reddish, 1928*b*). Hence in order to pass this test, these products must give a zone of at least 0.7 cm.

While the Serum-Agar Cup Plate Method does not duplicate practical conditions of use, it may properly be employed to compare with antiseptic preparations of known clinical value. Furthermore, the test does provide a means of measuring the penetration and inhibitory action of such antiseptics in the presence of blood serum. This test, like other laboratory tests, should be followed by practical and clinical studies.

#### FUNGICIDES AND FUNGISTATS

Fungicides and fungistats are divided into two general categories, (1) those intended for use only in the prevention and treatment of fungus infections, and (2) those designed and recommended for use on inanimate objects. Methods of testing have been developed for both and will be reviewed separately.

Laboratory methods of testing fungicides and fungistats have been concerned primarily with determining the value of such preparations for use in treating fungous infections of man and other animals. While many such tests have been suggested, none has been universally accepted, with the result that no standard method has as yet been adopted.

Probably the first laboratory method of merit was the one proposed by Schamberg and Kolmer (1922). In this method three of the most common pathogenic fungi were used as test organisms for determining both fungistatic and fungicidal activity. For determining fungistatic activity, the test organisms were inoculated upon Sabouraud's agar slants containing various concentrations of the compounds tested, then observed for inhibition of growth. In the fungicidal test, the same test organisms were grown on Sabouraud's agar, suspended in saline solution, mixed with different concentrations of the fungicides tested, and transfers made to slants of Sabouraud's agar at time intervals from 15 minutes to 24 hours.

This method, or modifications of it, has been used by many other workers in the field and is still quite generally employed. Myers and Thienes (1925) used essentially the same method in reporting on the fungicidal activity of certain volatile oils; Kingery and Adkisson (1928), Woodward, Kingery, and Williams (1933, 1934), and Kingery, Williams, and Woodward (1935) used certain modifications of the method of Schamberg and Kolmer in their extensive studies on volatile oils, stearoptens, and phenol derivatives. Klarman, Shternov, and Gates (1934*a*, 1934*b*) made use of essentially the same technique in their studies on the fungicidal activity of derivatives of parachlorophenol and orthochlorophenol. Stovall, Pessin, and Almon (1935) and Maplestone and Dey (1938) used modifications of this test in studies on thymol and certain other fungicidal substances. McCrea (1931) even suggested that the Schamberg and Kolmer method, or a slight modification of it, be adopted as the standard method for testing fungicides.

While this method of test has served a useful purpose in separating those substances which are fungicidal and may be effective for treating epidermophytosis, from those which may not have merit for the purpose, there are certain objections to the method which should be mentioned, especially since this procedure has been suggested as a standard test. The first objection to the test is that it does not in any way simulate practical conditions of use. In fact, the conditions created in this test are just the opposite of those met in practice. Suspending the organisms and spores in saline and shaking with glass beads to break up clumps and matted mycelia



before exposing to the action of the fungicide is quite different from the conditions of actual infection, where the organisms are closely matted and embedded in tissue. Testing the killing power of fungicides after breaking up the matted mycelium and spores of the test organisms is not logical nor does it simulate practical conditions. Also, no clinical evidence has been offered to show that this *in vitro* test is a satisfactory means of testing such fungicides. Since the method is an arbitrary laboratory test, there should be supporting clinical data to show that fungicides passing this test will be effective in practical use and that those not passing it will be ineffective in clinical practice.

Another serious objection to the method of Schamberg and Kolmer is that in transferring from the mixture of fungicide and culture, no precaution is taken to prevent inhibition of growth on the subculture slants. The method specifies that several loopfuls of the fungicide—culture mixture be transferred directly to slants of Sabouraud's agar. Since enough of the fungicide to exert a fungistatic effect may be and probably is carried over to the medium, false readings are likely to be made. Inhibition of growth may be interpreted as actual killing. These defects in the method are such as to make this method, in present form, inappropriate for testing fungicides intended for use in treating epidermophytosis.

Another method for testing fungicides that has been used by certain workers, is a modification of the phenol coefficient test. Emmons (1933) was among the first to use this technique in testing the fungicidal activity of common disinfectants. The highest dilutions of disinfectants which killed the test fungi in 5 and 30 minutes, were divided by the highest dilutions of phenol accomplishing the same result, giving a figure which was designated as the phenol coefficient. Similar technique was employed by Gomez-Vega (1935), Hesseltine and Hopkins (1935), and Dunn (1937).

Other quite different methods of testing fungicides have also been suggested. Strickler (1932) used collodion sacs containing suspensions of fungi for testing the fungicidal activity of elemental iodine. This method is well suited for the purpose, but would not be applicable as a method for testing other kinds of fungicides. Sharlit and Muskatblit (1932) and Sharlit (1935) used what they have designated as the "membrane method" which is best suited for testing volatile substances, such as thymol, etc., and water soluble fungistatic compounds. The fungicide to be tested is incorporated into collodion, which is then spread inside test tubes in which the agar medium is slanted. Test organisms are inoculated on surfaces of the slants and incubated. If the chemical substances are volatile or water soluble, enough may be absorbed into the

medium to inhibit the growth of the fungi used. This is a test for fungistatic activity, however, and is not a test for fungicidal properties. Therefore, it is not suitable for testing fungicides.

In their study on the fungicidal activity of pine oil, Smyth and Smyth (1932) made use of a new method of test which possesses more possibilities than any of those previously mentioned. The test organisms, fungi causing skin disease, are grown on Sabouraud's agar. Bits of the fungus growth, 1 mm square, are removed from the agar slant and are used as the test culture. After exposing these small squares of matted growth to the action of the fungicide for from 5 to 30 minutes, they are removed, rinsed in 95 per cent alcohol to remove excess fungicide, and then washed in saline to remove excess alcohol. The squares are then transferred to Sabouraud's agar and incubated at 20°C for 14 days. This method is more practical than others suggested for this purpose, and in addition, eliminates the possibility of fungistatic activity. The time periods of test are reasonable and logical.

Probably the most objectionable feature of this method is the difficulty of cutting the fungus growth into pieces 1 mm square and removing the agar from the growth. There is no need to use such small pieces of culture, and also, it is unnecessary to remove the agar. Instead of rinsing in 95 per cent alcohol, it is sufficient to simply rinse out the fungicide in water, by using a time period sufficient to remove it all. This would hardly be accomplished in the 10-second time period specified in the method. Furthermore, it is unnecessary to incubate the cultures for 1 to 2 months before use as test organisms. A much shorter time period is sufficient. Because of the small amount of culture used in this method, the test is not sufficiently severe to offer the usual margin of safety.

Fungicides recommended for treating fungus infections must of necessity kill or inhibit the different fungi causing them. In the past most investigators in this field have used more than one test organism and made efforts to use those fungi actually involved in epidermophytosis. This is not only desirable but necessary, since there is a certain amount of specificity in the fungicidal activity of many preparations used for the purpose. Somewhat typical of the test organisms employed are those used by Gould and Carter (1930), namely, *Trichophyton interdigitale*, *Trichophyton rubrum*, and *Trichophyton mentagrophytes*. These three fungi are the ones most commonly associated with ringworm of the feet and toes, or so-called "athlete's foot."

Burlingame and Reddish (1939) used five such test organisms. *T. interdigitale*, *T. rubrum*, *T. mentagrophytes*, *Epidermophyton floccosum*, and *Trichophyton rosaceum*. All of these organisms are

not present in every case of epidermophytosis and they are not always present in pure culture. But they do occur in this condition in pure or mixed cultures, and all of them must be killed if fungicides are to constitute an effective general treatment for the condition.

This test is simple, requires very little material, makes use of those organisms most commonly causing epidermophytosis, makes use of a solid medium in which the organisms grow most luxuriantly, uses a large inoculum, and satisfactorily avoids any bacteriostatic effect of the fungicides tested. The method is practical, as has been proved by clinical tests, and has been found satisfactory for the purpose of indicating which fungicides will and which will not prove effective under practical conditions of use.

It was found that those preparations which kill the test organisms within 5 minutes under the conditions of this test are clinically effective in the treatment of epidermophytosis. Clinical tests also showed that a preparation which did not pass this test—did not kill these test organisms in 30 minutes—was ineffective in the treatment of epidermophytosis. Thus it is apparent that this laboratory method of test is satisfactory for the purpose of indicating which fungicides will, and which will not, be clinically effective. It is also evident that this method is practical, simple, and simulates conditions as they exist in practice.

In early fungistatic tests, chemical substances were simply incorporated into the media used, either dextrose broth or Sabouraud's agar, onto which the test organisms were then inoculated, incubated, and observed for inhibition of growth. While this simple method is helpful in showing which substances will inhibit the growth of fungi and which will not, it offers no means of measuring penetration of the fungistatic substance. As stated previously, bacteriostatic activity of antiseptics is tested by means of the Serum-Agar Plate Method or the Agar Cup Plate Method. Under these conditions, test is made for penetration as well as inhibition of growth. Similar techniques may be employed in testing for the fungistatic activity of preparations that are applied for long periods of time. Ointments, salves, powders, etc., which are used in treating epidermophytosis may be tested for fungistatic activity by slight modifications of these methods.

The fungistatic test methods recommended by Burlingame and Reddish (1939) have been studied from the standpoint of dose-response curves and an "activity coefficient" has been determined (Oster and Golden, 1947). Dosage response curves were also obtained by a modification of the agar cup plate technique. As a standard for comparison, a 5 mm zone at 1 per cent concentration



of the compound tested is arbitrarily chosen as activity 1. Using this procedure, fungistatic agents can be compared on a numerical basis and be given numerical values. This constitutes a significant and valuable development in the field of fungistatic testing methods.

Relative fungicidal activity has also been determined by a modification of the above method, which includes use of 15-day-old fungus cultures, culture-fungicide contact of 1 minute, use of serum in the culture medium, and use of an acetone-water mixture for the washing phase of the test procedure (Golden and Oster, 1947). By use of this modification, it is possible to accurately classify fungicides by their minimal fungicidal concentration so that the relative merits of fungicidal compounds can readily be determined.

The accuracy of these methods in evaluating fungicidal and fungistatic activity has recently been demonstrated in a study of the antifungal properties of quinones and quinolines (Oster and Golden, 1948). These studies again demonstrate the value of the methods for their purpose.

While the phenol coefficient technique, or modifications of it, should not be used for testing fungicides recommended for therapeutic or prophylactic use on the body, such a procedure is quite applicable for fungicides used on inanimate objects. Klarmann, Shternov, and Costigan (1941) have recommended a modification of the phenol coefficient technique which is suitable for this purpose. An adequate factor of safety is included in this test so that the results may be interpreted directly. The number of fungi used in the test is at least 5,000 times the number found present in water in foot pans, and on shower and locker room floors (4 sq in). As just stated, this is a satisfactory method for testing fungicides for use on inanimate objects.

Although none of the above methods of testing fungicides and fungistats for use on living tissue have received official recognition by any recognized organization, a test for fungicides for use on inanimate objects has been so designated. The Association of Official Agricultural Chemists, after extensive studies, established a test method patterned after the phenol coefficient technique in essential details (Association of Official Agricultural Chemists, 1950).

In this test conidial suspensions of the test fungus—*Trichophyton interdigitale*—are added to dilutions of the fungicide, transferred to subculture media at the end of stated time periods, and incubated. Necessary precautions against fungistatic activity in the subcultures are provided. Suitable dilutions of phenol are included in the test but only as control on the resistance of the test culture. As just stated, this method is used for testing fungicides recommended for use on inanimate objects. It is not suitable as a test method for fungicides used for prevention or treatment of fungus infection.

## CHEMICAL STERILIZATION

Consideration of methods used for studying skin antiseptics leads quite naturally to a discussion of laboratory methods for determining the effectiveness of germicides for sterilization of surgical instruments. Reddish and Burlingame (1939) made a study of this problem and suggested a method which has been found suitable for the purpose. After determining the number of spores on dirty veterinary instruments, autopsy instruments used on laboratory animals which had died from anthrax and tetanus infections, and surgical and dental instruments, a test was devised in which spores of *Bacillus anthracis* were applied to safety-razor blades in such concentration as to provide for a margin of safety, that is, 100 times the maximum number of spores found on dirty veterinary instruments. After allowing the inoculated blades to dry, they were exposed to sterilizing agents for 10, 20, and 30 minutes at 20°C, rinsed in nutrient broth, then transferred to another tube containing 20 ml of nutrient broth. Both tubes of media were incubated at 37°C for 3 days. It was found that cold sterilization solutions regularly and successfully used in practice kill spores of *B. anthracis* by this test within 20 minutes. For this reason it was recommended that this time period be used for the practical sterilization of previously cleaned surgical and dental instruments.

In an extensive study of the chemical sterilization of surgical instruments under conditions in which the instruments were not first cleaned, Spaulding (1939) modified the above technique. Detachable surgical knife blades were inoculated with representative non-sporulating and sporulating infectious organisms in the presence of blood or pus. Four non-sporulating bacteria, three spore formers, and a yeast-like fungus were used as test organisms. The tests were conducted on both wet and dried blades and the time periods of exposure ranged from  $\frac{1}{2}$  to 10 minutes for the non-sporulating and from 5 minutes to 18 hours for the sporeforming organisms. Under these severe conditions of test, the vegetative forms were rapidly destroyed within a very few minutes, even when dried in the presence of body proteins, whereas the spore-formers were not killed within the 18 hours time period by some of the solutions tested. It is interesting to also note that one of the non-sporulating organisms, *Ps. aeruginosa*, was more resistant under the conditions of this test than was *M. pyogenes* var. *aureus*, and that one of the solutions tested required 30 minutes in order to kill the very large numbers employed. Also, the numbers of all the test organisms used on the blades were very large, this being one to supply an adequate factor of safety. While this method is

suitable for testing the effectiveness of germicides on unwashed blades, it is too severe for determining the value of such solutions under the usual use-conditions in which surgical instruments are washed before they are sterilized.

Tainter, Thronson, Beard, and Wheatlake (1944) made use of the method proposed by Reddish and Burlingame (1939) except that aluminum alloy strips, chromium plated on one side, were used instead of razor blades and 6 test organisms, 2 of them non-sporulating, were employed in the test. The tests were conducted on clean strips, the cultures being supplied directly to the surfaces without mixing with pus or blood. Under these conditions the results obtained confirm those reported by Reddish and Burlingame in the case of *B. anthracis*, although the time required to kill spores of *Bacillus subtilis* and *Clostridium botulinum* was longer. As might be expected, these results are quite different from those reported by Spaulding, since tests were conducted on clean metal surfaces in the absence of body proteins.

## GENERAL CONSIDERATIONS

The evaluation of the merits of disinfectants, antiseptics, and fungicides is not entirely based upon results of laboratory tests. While methods of testing are useful in reflecting germicidal and bacteriostatic properties, the final criteria must depend upon actual effectiveness when used in practice. Under some conditions, however, the results of *in vitro* tests can be interpreted directly after information from tests under use-conditions has been obtained.

In the case of phenol-like disinfectants this is comparatively simple. Use of the factor "twenty times phenol coefficient" is sufficient for calculating dilutions for use in practice. Other classes of compounds must be considered differently and it is expected that the results of laboratory testing of these germicides may be interpreted more accurately as further information as to their practical value is obtained.

Hunter (1943) emphasized this quite clearly in his discussion of the evaluation of antiseptics. He first warned against certain wide interpretation of results obtained by the Food and Drug Administration Methods of testing antiseptics. While these methods are used for determining germicidal and bacteriostatic properties, claims for effectiveness under conditions of use cannot be based solely on the results obtained by such laboratory tests. Hunter stated the situation quite clearly as follows: "The problem thus becomes one of fitting together the results of old established procedures with clinical observations and with the results of additional



tests, some recently developed and some not yet conceived, to form a complete picture of the properties of a substance under scrutiny." A similar view had previously been expressed by Browning (1934). It is evident that laboratory testing of antiseptics must be followed by practical *in vivo* tests, toxicity determinations, and by clinical tests under those practical conditions of use for which such preparations are recommended.

Essentially the same views were later expressed by Slocum (1950). He confirmed previous statements that "there is no single 'standard' or 'official' test for the evaluation of antiseptics" and stated further that "*In vitro* activity is not related directly to efficacy under conditions of use." While laboratory test procedures can and usually do indicate properties, clinical effectiveness under conditions of use constitute the final criteria.

The same is true of fungicides recommended for the treatment of epidermophytosis, as stated by Weidman, Emmons, Hopkins, and Lewis (1945). Here again laboratory testing is simply the first step in the process of evaluating the efficacy of fungicides for therapeutic use. Laboratory testing must be followed by clinical and toxicity tests.

The American Medical Association Council on Pharmacy and Chemistry (1943) advised the following tests in support of claims for any skin disinfectant submitted for consideration: *in vitro* tests in the presence and absence of serum; practical tests simulating conditions of use; *in vivo* tests by an animal method; animal tests for irritation and toxicity; clinical tests for efficacy and harmlessness; tests on bacteriostatic activity, as distinguished from germicidal activity. Much more than *in vitro* tests are required, not only in the case of skin antiseptics, but antiseptics generally and fungicides as well.

Certain other matters should be considered in connection with the laboratory testing of germicides. Interpretation of results obtained by these methods depends upon several important details. For example, the nature of the test organism is of vital importance, not only the kind of organisms used, but their resistance as well. This feature of our commonly used methods has been the subject of extensive study over a period of many years.

It is important that germicidal activity be determined on more than one test organism. Specifications for phenol resistance of representative test organisms have been established and should be extended. Ostrolenk and Brewer (1949) extended the list of suggested test organisms and reported the phenol resistance of representative strains. As emphasized by Brewer (1942) use of *M. pyogenes* var. *aureus* as the sole test organism does not provide the

margin of safety so important in such tests, especially in determination of germicidal activity of the synthetic detergents. Many germicides which kill certain other test organisms will not kill *Ps. aeruginosa*—further proof that use of a single organism is not desirable. In fact, it seems apparent that this resistant species (*Ps. aeruginosa*) should be used in all tests for germicidal activity.

It is also important that suitable culture media be employed for each of the test organisms used. This important detail has been recognized as a significant factor in the evaluation of results obtained by *in vitro* tests. It is, however, a factor that has often been overlooked by those of little experience in this field and has been an important cause of unreliable results.

As might be expected, peptone is important and has received special study. Shippen (1923) and later Reddish (1924) made a study on the effect of four widely used peptones on the resistance of *S. typhosa* and *M. pyogenes* var. *aureus*. Later Reddish and Burlingame (1938) extended this study to ten of the available peptones and compared the resistance to phenol of *M. pyogenes* var. *aureus* when grown in broth made from each. The most suitable peptone (Armour's) has consequently been specified for use in media employed in the testing of germicides.

Resistance of test organisms can be increased (Brewer, 1942) or lowered by departure from specified culture media. This can be done deliberately or by unintentional deviations which are not always easy to recognize. On account of this, some effort has been made to substitute synthetic and semi-synthetic media for the usual culture media employed for the purpose. With but one exception this has not proved satisfactory.

Klarmann and Wright (1945) developed a semi-synthetic medium containing Liver Vitamin B Concentrate U.S.P. XII in a synthetic substrate which, according to the authors, gave results in disinfectant testing quite comparable to those obtained with the media specified in the F.D.A. methods. Wolf (1945) suggested the use of a semi-synthetic medium containing acid-hydrolyzed casein and uracil as the main source of nitrogen as a substitute for broth media for maintaining resistance of *S. typhosa* and *M. pyogenes* var. *aureus*. It was claimed that this semi-synthetic medium was superior to broth media made from the peptone then available. Reddish, Wood, and Burlingame (1946) made a careful study of the semi-synthetic media of Klarmann and Wright and of Wolf and compared them to the media recommended in Circular 198 (1931). The resistance of *M. pyogenes* var. *aureus* grown in each of these media was determined, using phenol and Liquor Antisepticus N.F. as the test germicides.

Another synthetic medium has been suggested which appears to maintain the resistance of fourteen different test organisms over an extended period of time. Ostrolenk and Brewer (1949) made use of a medium containing only 2 per cent trypticase in distilled water. The reaction after autoclaving is pH 6.6 to 6.8, therefore is not adjusted, this being satisfactory for all the test organisms used. It is interesting to note that the resistance of *S. typhosa* and *M. pyogenes* var. *aureus* is the same against phenol in this medium as in regular broth. If the experience of others with this medium is comparable to that reported by Ostrolenk and Brewer, 2 per cent trypticase may well supplant broth as a medium for test cultures in the testing of antiseptics and disinfectants. Another valuable suggestion made by Ostrolenk and Brewer is that fluid thioglycollate medium be used for subculture after exposing the test organisms to germicides. This medium has also been found satisfactory by others in testing highly bacteriostatic compounds such as the mercurials.

Although *in vitro* methods of testing antiseptics, disinfectants, and fungicides are used primarily as screening tests, they are quite generally employed also in research on new compounds and new formulations. For example, the Cup Plate Method for testing antiseptic dyes is used quite generally as a screening test in research on antiseptics and fungistats. It is a quick means of determining quantitatively the possibilities of new compounds and formulations. Also, it is a quick and measurable means of determining stability of antiseptics and fungistats. The Agar Plate Method for testing antiseptic ointments is used for the same purpose, especially formulations in different ointment bases (Reddish and Wales, 1929; Foley and Lee, 1942; and others). In the case of fungistats, numerical values can be determined for comparative purposes (Oster and Golden, 1947).

The Serum-Agar Cup Plate Method is also useful for the evaluation of diffusibility of antiseptics in the presence of serum. Harris and Prout (1940) made use of this test for comparing the diffusion of antiseptics by this *in vitro* test and diffusion as observed in clinical practice. They determined the coefficients of diffusion of twelve germicides used in clinical practice and found that germicides having a high coefficient of diffusion, as determined by this method, were of value in obstetrics, that those having a low coefficient were of little value. They further state that, "The Agar Cup-Plate Method is recommended specially for the evaluation of germicides where their use will involve diffusion through colloidal material."



Rose and Miller (1939) and Miller and Rose (1939) made extensive studies on the Agar Cup-Plate Method and found, as might be expected, that various factors may influence results. Although the conditions of conducting the Agar Cup-Plate Method are specified, the authors made a study of the effects of varying these conditions. They emphasized the importance of strict adherence to the details of the method, and added some minor additional details. They also found that plain agar does not affect the antiseptic properties of mercury compounds, but that the presence of blood in the medium decreases the antiseptic potency of such compounds, according to the amounts present. This is, of course, an important consideration in the testing of mercury compounds.

*In vitro* germicidal tests are also employed as screening tests in research on germicidal soap. Although Walker (1931) reported that soap is germicidal against pneumococci, streptococci, and certain other weak organisms, soap is not germicidal when applied to the more resistant skin organisms (Reddish, 1930; Klarmann and Shternov, 1941; and others). The *in vitro* tests commonly used for this purpose are sufficient for separating the obviously ineffective soaps from those containing germicides of potential value (Cade, 1935). If by *in vitro* test the soaps containing germicides are found to be germicidal by these severe tests, they are then submitted to practical tests under conditions of use (Maglio, 1948), and others.

The influence of wetting agents on the germicidal value of antiseptics and other germicides can readily be studied by such *in vitro* tests (Fisher 1942). While no single wetting agent could be depended upon to enhance the germicidal activity of all kinds of antiseptics, the laboratory methods employed by Fisher were useful in determining the degree of such activity as a result of the use of wetting agents in the formulations. This is especially important since it is not always possible to adjust antiseptics to specified pH levels for the purpose of increasing germicidal activity, whereas the use of wetting agents for this purpose is comparatively simple. Ordal, Wilson, and Borg (1941) had previously shown that the addition of wetting agents to buffered solutions of phenolic compounds increased the germicidal activity of such solutions. The specific effect was shown to be a function of the character and concentration of the wetting agent employed.

However, as might be expected, results by the Agar Cup-Plate Method do not correlate results obtained by the phenol coefficient method. Tobie and Ayres (1944), found that use of either the Agar Cup-Plate Method or the phenol coefficient test could not be considered alone in determining the effectiveness of antiseptics and

germicides. The Cup-Plate test gives information as to diffusibility and bacteriostatic activity and the phenol coefficient test shows the extent of germ killing power. It is further indicated that new compounds might be rejected if only one of the methods were used, so both must be employed in evaluating the possibilities of such compounds.

Since the various methods of testing germicides often give results quite different one from the other, it becomes necessary to evaluate them critically. The interpretation of even well-known and widely used methods is a matter of considerable importance (Reddish, 1947*a*, 1947*b*, 1948, 1950). The evaluation of these methods must always be considered in relation to their interpretation and the conditions under which such preparations are used.

While it would be desirable to develop methods of test which required little if any such interpretation, this has not as yet become possible. Various authorities in the field have recently suggested changes in present procedures for the purpose of more nearly approaching this ideal (Cade, 1937; Wolf, 1946; Cade, 1947*a*, 1947*b*, 1947*c*, 1949; Rahn, 1947; and others). However, many of the present tests may be used with the assurance that, *with proper interpretation*, they can be employed for the purpose of indicating the value of disinfectants, antiseptics, and fungicides under actual conditions of use.

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## 4

# METHODS OF TESTING ANTISEPTICS

SEVERAL methods are available for testing antiseptics which may be used for specific purposes. Some of these are used primarily for screening, while others are employed for determining bactericidal spectrums, penetration, performance under use conditions, and for other purposes. There are also two official test methods which are specified for the testing of certain official preparations. However, there are no standard methods for testing different kinds of antiseptics which are recognized as official government tests for the purpose.

The phenol coefficient method for testing disinfectants was for many years used for testing liquid antiseptics as well. In fact this situation continued until Shippen (1923) began the use of *Micrococcus pyogenes* var. *aureus* in testing disinfectants which were also recommended for antiseptic uses. A modification of the phenol coefficient technique was used for the purpose. Later Reddish (1925, 1927) further perfected the method of test which was then used for evaluating antiseptics. A few years later, other procedures were developed for testing different kinds of antiseptics other than solutions (Reddish, 1928, 1929).

These methods were later set forth and prescribed for use in the examination of antiseptics by the U. S. Food and Drug Administration (Circular 198, 1931). While these methods were not actually designed as official test procedures, they were used for control purposes by the Food and Drug Administration for many years and widely employed elsewhere. These methods are still useful for the purposes for which they were designed, that is for control and for screening purposes, and for determining the antiseptic activity of new compounds and new formulations. They are still employed for these purposes with the full understanding that they are not official government methods. Since these methods are still useful for the



purposes specified, they are outlined here as originally described by Reddish (1929) so that the designation "Food and Drug Administration Methods" will not be further encouraged, which is in keeping with the expressed wishes of this governmental agency.

In developing laboratory methods of testing antiseptics, the conditions of test should either simulate practical conditions of use or should be based on known clinical values. This basic reasoning has been employed in developing methods for testing the various classes of antiseptics. Since *M. pyogenes* var. *aureus* is the most common cause of suppuration and is the most resistant of the non-sporing infectious microorganisms, it is the logical organism for use in testing antiseptics, at least for control purposes and in preliminary screening tests. It must be emphasized that laboratory tests of any kind are but the first steps in any study of the effectiveness of antiseptics. These tests must then be followed by performance tests under conditions of use and then by clinical studies.

The importance of laboratory testing, however, must not be minimized, since it is necessary in any study of this class of drugs. Present methods of test are designed to give valuable information as to antiseptic action under conditions which are either exaggerated or which simulate to some degree the conditions under which they are used. The methods developed by Reddish (1929) are outlined here for the purposes indicated.

## REDDISH METHODS OF TESTING ANTISEPTICS

### SOLUBLE AND LIQUID ANTISEPTICS

Liquid antiseptics which are applied for short time periods, that is for use on cuts, scratches, minor wounds, gargles, etc. are tested by the following method:\*

*Test Organism.*—*M. pyogenes* var. *aureus*, Food and Drug Administration strain No. 209 (American Type Culture Collection No. 6538).

*Medium.*—Beef extract (Difco) 5 g, peptone (Armour) 10 g, sodium chloride 5 g, distilled water 1000 ml. Boil for 30 minutes to dissolve; adjust to pH 6.8 with normal NaOH or saturated aqueous  $\text{Na}_2\text{CO}_3$ ; boil 10 minutes; filter through paper; and make up to original volume. Add 10 ml into 19 x 150 mm unlippered test tubes, plug with cotton, and sterilize in the autoclave at 15 lbs pressure for 30 minutes. Nutrient agar for stock cultures is made by adding 1.5 per cent Bacto-Agar (Difco) to this broth base adjusted to pH 7.4.

\* Adopted as the official test for antiseptic value for Antiseptic Solution, N.F. (The National Formulary, 9th. ed., 1950, p. 57-58.)

*Stock Culture.*—The stock culture is transferred on agar slants of the above composition each month and stored at 50°F.

*Test Culture.*—The test culture is prepared by transferring from the agar slant stock culture into 10 ml of the above broth medium and transferred and incubated at 37°C for 3 consecutive days. This culture must be tested for resistance to phenol and must resist 1 to 80 phenol for 5 minutes and 1 to 90 phenol for 15 minutes at 37°C. Such a culture is suitable for use in testing antiseptics.

*Medication Tube.*—Unlipped test tubes 25 x 150 mm plugged with cotton and sterilized in the hot air oven at 170°C for 1 and ½ hours are used for mixing the culture with the antiseptic in the test.

*Temperature of Test.*—The antiseptic and test culture must be warmed in a water bath to 37°C and held at this temperature during the period of the test.

*Inoculation Loop.*—A 4 mm loop of platinum wire, U. S. No. 23 B and S gage, 1½ to 3 in long, set in suitable holder such as aluminum or glass rod 0.5 cm in diameter, is used to transfer from the antiseptic-culture mixture in the medication tube to 10 ml of sterile broth in the subculture tubes. The loop and rod are flamed before each transfer, which is made under aseptic conditions.

*Incubation.*—The subcultures are incubated at 37°C for 48 hours.

*Dilutions.*—Any series of dilutions which may be required are made in sterile distilled water under aseptic conditions, or the antiseptic may be tested undiluted.

*Method of Conducting Test.*—Five ml of the antiseptic, either undiluted or diluted as desired, are placed into sterile 25 x 150 mm test tubes and warmed to 37°C in a water bath. The 24-hour broth culture of the test organism, after vigorous shaking, is allowed to warm in the same water bath for 5 minutes. Five-tenths ml of this culture is removed by means of a 1 ml graduated pipette and added to the 5 ml of antiseptic and mixed by slight agitation. Transfers are then made from the mixture of culture and antiseptic into 10 ml of sterile broth by means of a sterile 4 mm loop at intervals of 30 seconds, 1, 2, and 5 minutes. These transfer tubes are then incubated at 37°C for 48 hours. At the end of the incubation period these broth tubes are observed for evidences of growth.

Since there is always the possibility that enough of the germicide may be carried over into the subculture broth to inhibit growth of the test organism, false results may often be indicated. For this reason it is necessary to determine whether inhibitory concentration of germicide is present in the broth showing no growth by reinoculating these tubes with a fresh 24-hour broth culture of

the test organism by means of a sterile loop and reincubating at 37°C for 24 hours. If growth occurs after this inoculation it means that no inhibitory action has occurred and that failure of growth during the first incubation shows that the test organisms had been killed. In case no growth occurred after the second inoculation the test must be repeated using 250 ml of broth in a flask in place of 10 ml to avoid inhibitory action of the antiseptic in the subculture.

The information desired is the concentration of the antiseptic required to kill *M. pyogenes* var. *aureus* under the conditions of the test within 5 minutes. If a preparation does not pass this test within this time period, it is considered not sufficiently germicidal to be classified as an antiseptic. Those preparations that do pass the test within 5 minutes or less, must then be submitted to further study under practical and clinical conditions.

*Interpretation of Results.*—The proper interpretation of results obtained by the above method is quite important. In a sense it is only a “screening test,” intended to separate preparations which are not actively germicidal from those which are positive and quick-acting germicides. This is important, however, and is sufficient justification for the test.

On the other hand the results can properly be interpreted indirectly by comparison with results obtained with other liquid germicides of known merit. For examples, 2.0 per cent phenol and 70 per cent alcohol are germicides of proved clinical value. They have both been used in clinical practice with satisfactory results. When tested by the above laboratory procedure they both pass the test within 5 minutes but they do not pass the test when diluted 1 + 1 with water. This means that liquid antiseptics which give the same results by this laboratory test may be considered equal in germicidal activity to these two widely used germicides.

## ANTISEPTIC OINTMENTS

Antiseptic ointments must be tested by a method quite different from the above test for soluble and liquid antiseptics. The reason for this is that ointments when used remain in contact with infective bacteria and exert their activity for long periods of time. They render the bacteria innocuous by simply preventing their growth, thereby avoiding infection. This being the case a method is employed which tests for penetration and inhibitory (bacteriostatic) activity. The test is known as the Serum-Agar Plate Method and is conducted as follows: *M. pyogenes* var. *aureus* (F.D.A. Strain No. 209) is grown in broth of the above composition, transferred and incubated at 37°C for 3 consecutive days, and tested for resis-



tance to phenol. Using the method outlined above, the test organism must resist 1 to 80 phenol for 5 minutes and 1 to 90 for 15 minutes at 37°C. One-tenth ml of a 1 to 100 dilution of such a resistant culture is then added to 20 ml of nutrient agar of the above composition which has been melted and cooled at 45°C. Ten per cent (2.0 ml) of sterile normal horse serum (without preservative) is then added, mixed thoroughly with the inoculated agar by pouring back and forth into another sterile empty tube, and then poured into a sterile 9 cm Petri dish with unglazed clay top, and allowed to cool at room temperature. After the agar has hardened the ointment is placed into intimate contact with the surface of the agar in the form of a streak. This is done by melting a small quantity of the ointment by warming slightly over a small flame in another empty sterile Petri dish. Care must be exercised in melting the ointment and this is done by touching the bottom of the Petri dish to the back of the hand at intervals so that it will not be heated too much. The melted ointment is then streaked over a small surface of the agar by means of a sterile glass rod. A streak  $\frac{1}{4}$  inch wide and  $1\frac{1}{2}$  inch long is sufficient and should be placed in the middle of the upper half of the Petri dish. A similar streak of 'Vaseline' is placed on the surface of the agar in the lower half of the same plate to serve as control. The plate is then incubated at 37°C for 48 hours.

If the preparation tested is antiseptic, a zone of clear agar will be noted surrounding the streak. The width of the clear zone will indicate the penetration or diffusibility of the antiseptic agent. The measurement of the zone must be made from the outer edge of the ointment streak to the inner edge of the zone. When the edge of the zone is hazy and where a few colonies appear within the area, the measurement must be made up to the colonies nearest to the ointment streak. If the preparation is not antiseptic, there will be no clear zone around the ointment but colonies of the test organism will be observed adjacent to and even under the streak. Since petrolum jelly is not antiseptic the colonies of the test organism will completely surround the streak since growth of the organism is not inhibited.

This test demonstrates not only the presence or absence of an antiseptic ingredient in the ointment, but also determines the relative amount of such active ingredient. It also indicates the diffusibility of the antiseptic and also whether or not it is counteracted by blood serum. In addition the results will indicate whether the antiseptic has a greater affinity for the base than it has for the serum and water contained in the serum-agar medium. It will also prove whether the antiseptic is effective against *staphylococci*. Experience has shown that antiseptics which inhibit *M. pyogenes*

var. *aureus* by this test are even more effective against most other infectious micro-organisms.

*Interpretation of Results.*—The purpose of this method of testing antiseptic ointments is to determine whether and to what extent the antiseptic ingredient will leave the ointment base in the presence of moisture and blood serum in semi-solid substrate. Another purpose is to measure the effect of blood serum on the activity of the antiseptic since many antiseptics are counteracted by organic matter. The antiseptic should penetrate the semi-solid serum in sufficient concentration to inhibit the test organism in an area at least 0.2 cm wide.

The size of the zone of inhibition given by this method is important and has a direct bearing on the possible clinical effectiveness of the ointment being tested. Since Ammoniated Mercury Ointment, U.S.P. has been widely and successfully used as an antiseptic ointment by the medical profession and others for a great many years, it is used as the standard of comparison in the test for antiseptic ointments. A clear zone of inhibition of 0.2 cm is given by this standard antiseptic ointment (Reddish and Wales, 1929).

#### WET DRESSING, DYES, POWDERS,\* OILS, ETC.

Liquid antiseptics used in wet dressings, antiseptic dyes, powders, oils, etc. for continuous application are tested by a method similar to that just described for antiseptic ointments. This method is known as the Serum-Agar Cup Plate Method.

The serum-agar is prepared as described above and inoculated with *M. pyogenes* var. *aureus* in the same way. Twenty ml of this inoculated serum-agar is poured into a sterile Petri dish with unglazed clay top. After the agar has cooled, a well is made in the middle of the plate by means of a 1.5 cm cork borer and sealed with 1 or 2 drops of melted agar. One-half ml of the liquid antiseptic to be tested is placed into the cup and the plate incubated at 37°C for 48 hours. The clear zone of inhibition indicates the degree of penetration and antiseptic action of the preparation tested. The zone is measured from the edge of the cup to the nearest colonies in the inner edge of the zone.

When antiseptic dye solutions are tested by this method they are placed into the cup in the same manner as liquid antiseptics used for wet dressings. Antiseptic powders and oils which are recommended for continuous contact, that is applied to wounds or infected areas for long periods of time, are added to the cup

\* See also the official test for antiseptic value for Compound Zinc Sulfate Powder, N.F. (The National Formulary, 9th. ed., 1950, p. 575.)

in the same manner. In the case of powders, the cup is filled to the surface of the agar while the oils are added in 0.5 ml quantity. The incubation period and measurement of the zone of inhibition is the same as for liquid antiseptics and solutions of dyes.

*Interpretation of Results.*—The interpretation of the results obtained with these preparations is comparable to that given above for antiseptic ointments except that the zone of inhibition acceptable is somewhat larger. An antiseptic dye solution of known merit which has been widely and successfully used in surgery and first-aid gives a zone of 0.7 to 0.9 cm by this method. In order to pass this test, such products should give a zone of at least 0.7 cm, and powders and oils 0.4 cm.

While the Serum-Agar Cup Plate Method does not simulate practical conditions of use, it may properly be employed to compare antiseptic preparations of known clinical value. Furthermore, the test does provide a means of measuring the penetration and inhibitory action of such antiseptics in the presence of blood serum.

It must be emphasized again that these very simple *in vitro* tests constitute only the first step in determining the actual value of these antiseptic preparations for the purposes intended. Additional performance tests under conditions of use and clinical studies must then be made before claims can be made for effectiveness when used in practice. The results of laboratory, practical, and clinical tests are used to show germicidal or inhibitory properties of these drugs and their efficacy when applied under conditions of use.

Test organisms other than *M. pyogenes* var. *aureus* may be used in the above test procedures provided culture media suitable to each be employed in the tests, as has been recommended by Reddish (1927), Philbrick (1929), and many others. Ostrolenk and Brewer (1949) have recommended the use of several different test organisms because of the "... impossibility of adequately determining germicidal activity with one representative test organism by a single standardized type of test." Because of this and other desirable features of the procedures recommended by Ostrolenk and Brewer, the test method is presented here in detail.

## OSTROLENK AND BREWER BACTERICIDAL SPECTRUM METHOD

*Test Organisms.*—*M. pyogenes* var. *aureus*, *Micrococcus pyogenes* var. *albus*, *Streptococcus fecalis*, *Corynebacterium hofmanni*, *Pseudomonas aeruginosa*, and other organisms suitable for the purpose.



*Media.*—The test organisms are grown in 10 ml unadjusted 2 per cent trypticase, pH 6.6 to 6.8 after sterilization. Subcultures after exposure to germicidal agents are transferred to 15 ml fluid thioglycollate medium.

*Method of Test.*—All test cultures are transferred daily by means of a 4 mm loop and incubated at 37°C. The phenol coefficient technique is employed, that is 0.5 ml of 24-hour test culture added to 5 ml of antiseptic at 20°C and 37°C for 5, 10, and 15 minutes and transferred by 4 mm loop to 15 ml thioglycollate medium and incubated at 37°C for 4 days. The phenol resistance of the test cultures is determined and only test cultures of specified resistance are used. As additional check on the cultures, bacterial counts are made at frequent intervals on 24-hour cultures in trypticase broth.

There are certain obvious advantages in this method of testing: (1) a variety of test organisms of different degrees of resistance are employed; (2) the medium for the test culture is simple and assures uniform resistance of the test organisms; (3) the use of thioglycollate medium for subcultures after exposure to germicides assures growth of all surviving organisms and eliminates or minimizes bacteriostasis in subcultures; and (4) offers standards of resistance to phenol of each test organism together with total counts expected in the test cultures. For these reasons this method should be employed as a primary laboratory test for germicidal activity of liquid antiseptics.

Another useful and promising procedure for testing antiseptics is the "semi-micro" method of Klarmann and Wright (1948, 1950). Developed originally for testing quaternary ammonium disinfectants, it is equally suited for testing antiseptics, especially those containing quaternaries and similar compounds. Since this method offers certain advantages for the purpose, it is given here in detail.

## KLARMANN AND WRIGHT "SEMI-MICRO" METHOD FOR EVALUATING ANTISEPTICS

*Test Organism.*—*M. pyogenes* var. *aureus*, Food and Drug Administration, No. 209.

*Medium.*—Beef extract (Difco) 5 g, peptone (Armour) 10 g, sodium chloride 5 g, distilled water, 1000 ml, adjusted to pH 6.8.

*Procedure.*—Pipet 0.05 ml of a 24-hour broth culture of *M. pyogenes* var. *aureus* onto the bottom of sterile 25 x 150 mm test tube and place in the water bath at 20°C. Five-tenths ml of the antiseptic, previously kept at 20°C, is added and mixed with the culture. Ten minutes later 20 ml of broth or blood (10 per cent) broth is added to the antiseptic-culture mixture and incubated at 37°C for 48 hours.

The principal advantage of this method is that it avoids sampling errors of the loop transfer method after exposure of culture to germicide. This is especially important in testing quaternary ammonium compounds or formulations containing these or similar components. Also the method tests for complete kill of all the organisms used in the test and not of the number removed by loop transfer. This method is limited to liquid antiseptics.

The laboratory testing of antiseptic soaps has been a problem for many years and there is still no satisfactory laboratory method. The "Filter Paper Method" recommended by Reddish (1929) has been used to some extent, but because of serious defects and uncertainties in interpretation this procedure has been largely discarded. In the meantime Price (1938) devised a highly satisfactory *in vivo* method for determining bacterial reductions on the skin as the result of mechanical cleansing designated as "degermation." Although the technique was designed to count skin bacteria before and after scrubbing with soap and brush, Price (1939) used the same procedure for studying the effect of alcohol as a degerming agent when applied to the skin. The method also served to separate and enumerate the so-called "transient" and "resident" flora of the skin under various conditions. The Price technique has been widely used for these purposes, especially for determining degermation of the skin following the use of antiseptic soaps and synthetic detergents.

#### PRICE TECHNIQUE FOR DETERMINING DEGERMATION OF THE SKIN

*Method of Test.*—Several sterile enamel 5L basins are employed; 2000 ml sterile water is added to each basin; the hands and forearms are scrubbed with soap and sterile brush for 35 seconds, dividing the time equally between each hand and arm, and 15 seconds allowed for rinsing the hands and arms into the basin; 1 ml and 0.5 ml are immediately plated into nutrient infusion agar and incubated at 37°C for 48 hours; the same process is repeated in the remaining basins; total bacterial counts are calculated for each basin and the degerming effect determined by percentage reduction figures. The same technique, with slight modification to prevent bacteriostasis, may be used for determining the degerming effect of antiseptic soaps and synthetic detergents.

When the technique is used for testing the degerming effect of such germicides as alcohol which are not highly bacteriostatic no modification is necessary. However, when soaps and detergents containing highly bacteriostatic compounds are employed in the

test, the platings from the wash waters in each basin may be so highly inhibitory that no growth will occur. In order to avoid this effect the hands and arms must be rinsed in fresh sterile water following the scrubbing in each of the wash basins, in which case the rinse water only is plated. Cade (1950) and others have used this modification with satisfactory results in studying the degerming effects of various antiseptic soaps and detergents containing highly bacteriostatic compounds.

When the skin is broken and when prevention of infection is involved, another quite different method is employed. For this purpose the "infection-prevention" test devised by Nungester and Kempf (1942) has been found most satisfactory. The test is designed especially for evaluating antiseptics applied to broken skin such as cuts, scratches, abrasions, etc. which are subject to direct infection. Although the method has certain limitations and is somewhat lacking in detailed specifications, it is the best procedure available for the purpose.

#### NUNGESTER AND KEMPF "INFECTION-PREVENTION" TEST FOR SKIN ANTISEPTICS

The test organisms employed are a strain of type I pneumococcus and a hemolytic streptococcus. Mice are first anesthetized and the tip of the tails inoculated with the test cultures by means of cotton swabs. The tip of the tail is then dipped into the antiseptic under test and allowed to act for 2 minutes. At the end of this time period  $\frac{1}{2}$  inch of the tail is removed and inserted into the peritoneal cavity and the incision closed. The animals are observed daily for 7 to 10 days and the heart blood of dead animals streaked on blood agar. If the animals survive, the antiseptic under test is considered satisfactory as a skin antiseptic, but if the animals die it is evident that the antiseptic has been ineffective. The nature of the test is such that pneumococci and streptococci must be used as test organisms, but since both are weaker than the staphylococci the test is not sufficiently severe to prove effectiveness against those organisms which are the most common cause of suppuration. Otherwise the test is quite satisfactory and useful in studying skin antiseptics.

Neither this method nor the one developed by Price provides for determination of toxicity of antiseptics. Spaulding and Bondi (1947) have made use of the Nungester and Kempf method in combination with a toxicity test to provide for an "Infection Prevention-Toxicity" method. Antiseptics applied to the unbroken and broken skin may reach deeper tissues either by absorption through the skin



or directly through the broken skin. The method includes two separate tests, one for infection-prevention and the other a test for toxicity.

### SPAULDING AND BONDI "INFECTION PREVENTION-TOXICITY" METHOD

The method first determines the highest dilution of antiseptic preventing infection in at least one-half of the mice, which is designated as Infection-Prevention/50 (IP/50). The toxicity test determines the greatest concentration of the antiseptic which fails to kill one-half the mice in the absence of the test organisms, which is designated as Toxicity/50 (T/50). IP/50 divided by T/50 gives a figure designated as IP-T/50 which measures the difference between bactericidal activity and toxicity. Therefore the higher the index value of an antiseptic agent the more satisfactory it should be for clinical use.

### GENERAL CONSIDERATIONS

Since there are several methods of testing antiseptics, any one or all of the procedures outlined may be used, according to the information desired. It has become common practice to employ a combination of test procedures in order to study the various factors involved.

Salle and Catlin (1947) recommended the use of 6 different tests in order to determine a profile evaluation of antiseptics. Reddish (1950) suggested the following panel of tests: (1) Reddish Method for germicidal activity, using *M. pyogenes* var. *aureus* at 37°C; (2) the Agar Cup Plate Method, with and without 20 per cent blood serum, for determining bacteriostatic activity and effect of organic matter; (3) infection-prevention method of Nungester and Kempf; (4) infection-prevention/toxicity index method of Spaulding and Bondi; (5) irritation tests on animals, such as instillation into the eyes of rabbits; (6) tests for germicidal spectrum by the method suggested by Ostrolenk and Brewer; and (7) the Oster and Golden (1947) modification of the Burlingame-Reddish (1939) method for determining fungicidal and fungistatic activity. Such tests are useful in estimating antiseptic and fungicidal activity and in some degree in predicting possible performance under conditions of use.

Interpretation of results of certain *in vitro* tests has been favorably discussed by Reddish (1947). Also a comparative study of laboratory and practical tests (Reddish, 1936) resulted in the observation that antiseptics which pass the *in vitro* test for germi-

cidal activity were effective in killing bacteria under conditions of use *in vivo*. These results give added assurance that this laboratory method of testing liquid antiseptics is satisfactory as a means of estimating or at least indicating effectiveness for practical use. This was further confirmed by clinical tests on over 3,000 human subjects in which the oral use of such an antiseptic resulted in significant reductions in the number, severity, and duration of upper respiratory infections.

There are many other instances in which performance tests confirm results of laboratory tests of antiseptics. Shay (1951) has shown that hexachlorophene in synthetic detergents when used in practice is effective in reducing skin bacteria to a significant degree. Cade (1950) proved performance effectiveness of the same compound under conditions of use, as have Maglio (1948) and others. This compound was found to be germicidal under *in vitro* laboratory conditions before being submitted to practical use performance tests.

One of the most extensive studies on the evaluation of germicides under *in vivo* conditions is that of Prombo and Tilden (1950). A total of 39 germicidal solutions were submitted to the infection-prevention test of Nungester and Kempf using the pneumococcus as the test organism and another series of 20 using *S. pyogenes*. Activity against the tubercle bacillus was also studied by *in vivo* tests with 12 germicides. As might be expected there was considerable variation in effectiveness of the various germicides under *in vivo* test conditions, but it was shown that there was a correlation between known germicidal activity as determined by *in vitro* laboratory tests and results obtained by *in vivo* tests.

The results of laboratory tests must, of course, be interpreted according to the nature of the antiseptic and the conditions under which it is used. In this connection it is well to emphasize the fact that when used clinically antiseptics continue to act for varying periods of time. In the case of organic mercurials, for example, this is usually an extended period, whereas certain other antiseptics act quickly. Such quick-acting germicides, however, are not necessarily better than the slower-acting organic mercurials. They act against infection in their own way under clinical conditions of use.

Some antiseptics which are considered only bacteriostatic in their activity are often slow-acting germicides. This has been proved quite clearly by Price (1950) who showed that certain compounds in supposedly bacteriostatic concentration were actually germicidal when exposed to test organisms for long periods of time. This also has a direct bearing on *in vitro* and *in vivo* methods of testing and must be given due consideration in the use of and

interpretation of results from these tests. For this reason quantitative tests may be of more value than qualitative tests since the interpretation of results would be more valid and would more closely correlate performance under use conditions. However, as again emphasized by Price, results of such tests must not be interpreted directly, but must be followed by performance tests under conditions of use and by actual clinical tests.

Cade (1949) also emphasized the importance of bacteriostatic potency of chemicals used as antiseptics and has shown that there is an overlapping between germicidal and bacteriostatic activity. Bacteriostasis, therefore, does not necessarily imply inhibition and nothing more, but under certain conditions a mild or slow form of germicidal activity may be involved. This is proved quite clearly in the use of preservatives in food, pharmaceuticals, etc. in which bacterial counts decrease over a period of time due to the action of the preservative.

The role of antagonism or bacteriostasis in laboratory methods of evaluating antiseptics is obvious. Lawrence (1950) and Klarmann (1950) have discussed this subject rather fully and have specified the use of inactivators to eliminate inhibition of test organisms in *in vitro* test procedures. This is in keeping with recommendations of many others who are fully aware of the importance of this detail in the testing of germicides generally.

While these and other refinements are helpful in the laboratory testing of antiseptics, the *in vitro* test procedures are for preliminary evaluation only. They constitute the first step in the study of antiseptics and must of necessity be followed by practical performance tests and clinical study. As preliminary tests, these laboratory procedures are useful and necessary in any evaluation of antiseptics, and may be used with confidence for the purpose.

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## 5

# METHODS OF TESTING DISINFECTANTS

## INTRODUCTION

EXPERIENCE has shown that a chemical analysis does not give sufficient information for evaluating the effectiveness of germicidal preparations as disinfectants. Germicidal activity is not solely a property of chemical composition but is greatly affected by such physical factors as solubility, miscibility, degree of colloidal dispersion, ionization, surface tension, and other less well-defined properties. Thus, disinfectants can be compared one with another and evaluated for various applications only by supplementing analytical data on composition with the results of bacteriological tests.

It should be commonly recognized that no single bacteriological test method can be expected to be suitable for determining the efficiency of all germicidal chemicals in all of the disinfecting uses for which they may be recommended. Therefore, the problem of testing disinfectants resolves itself into, (1) the selection or the development of a method which will provide a useful result, (2) careful application of the method selected or developed, and (3) the proper interpretation of the result obtained.

If useful data are to be obtained, it is essential that the test method developed or selected, (1) be adequately controlled, (2) be precise enough so that results can be uniformly reproduced, and (3) yield data that can be accurately interpreted in terms of practical disinfecting values.

The wide variations encountered in the resistance of different species of bacteria, between strains of individual species and between cultures of individual strains depending upon such factors as media composition, time and temperature of incubation, routines of stock culture maintenance, and the like make it essential that the resistance of each test culture employed be determined at the

time the test is made, employing a known germicidal chemical as a standard. Only when this is done, and conventional checks are made on culture viability and the sterility of the media and apparatus employed, is it possible to claim that a standard for comparison has been established. This procedure can be identified, therefore, as a primary requisite for adequate control.

The development of any bacteriological test method with sufficient precision for use in referee work is always a difficult and tedious task. While many methods for testing disinfectants have been proposed, relatively few of them have received sufficient attention on this score. Meticulous standardization of apparatus, equipment, media composition and reaction, stock culture and test culture maintenance routines, and manipulation detail are as essential as the preparation of exact concentrations of the germicide to be tested, accurate measurement of germicide and test culture volumes, exact temperature control, and accurate timing in obtaining a precise result.

While it must be acknowledged that the final criterion on the effectiveness of a disinfectant is the demonstration of disinfecting efficiency under conditions of practical application, the primary responsibility for the evaluation and classification of disinfectants belongs to the laboratory. In the laboratory and only there is it possible to establish control over the many variable factors encountered in actual performance to the degree necessary for elimination of fundamental uncertainties in the conclusions that may be drawn. If the controlled laboratory test fails to yield a result that can be employed to provide a reliable index to practical value, then it becomes necessary to classify the test as inaccurate. Conversely, the interpretation of the laboratory result in terms of practical disinfecting value can be identified as a requirement for accuracy.

## HISTORY

Koch (1881) loaded threads with anthrax spores, and then exposed them for varying periods of time in solutions of chemicals to determine their value in destroying this infectious organism. After exposure, the threads were washed and implanted in nutrient gelatin or used for the inoculation of animals to determine the survival of the test organism. Since the effective concentrations of the germicides found in this test were considered as potentially effective in actual use, the method can be identified as a procedure intended as a direct measure of disinfecting value against a specific spore-forming infectious agent.



This method was adopted by Delepine (1907) and others for use in measuring the germicidal activity of chemicals against various non-spore-forming bacteria. Delepine (1907) called attention to the desirability of using this method to confirm results obtained by other procedures where unprotected bacterial cells were exposed to solutions of chemicals. The most common objections raised against its use have been that, (1) the exposed thread carrier, in spite of any washing procedure which may be applied, tends to carry over into the subculture medium bacteriostatic quantities of the germicide, and (2) the test organisms tend to be protected against the action of the chemicals by partially dried or dried films of organic matter originating in the medium in which they are grown. Modifications intended to provide more adequate control and overcome these deficiencies have continued to be used on special occasions to provide useful results and Ortenzio, Stuart, and Friedl (1953) described a procedure of this type as being the most suitable for determining the practical sporocidal value of germicidal preparations.

Krönig and Paul (1897) proposed a garnet method in which the test bacteria were dried on the surface of little garnets of uniform size prior to exposure in the test solution for varying periods of time. These could then be washed and treated with chemical reagents to neutralize and prevent a carry-over of bacteriostatic quantities of the disinfectant into the subculture medium more effectively than was found possible with the thread carrier proposed by Koch (1881). As was the case with the thread method of Koch (1881), the results were interpreted directly in terms of practical disinfecting concentrations. In line with this interpretation, Mallmann and Hanes (1945) standardized a glass rod carrier method and proposed its application as a direct measure of the maximum safe use-dilution of disinfectants. In subsequent studies, Mallmann and Leavitt (1948) found that this procedure provided a safe index to the practical disinfecting value of germicides commonly employed by veterinarians. Following their reports, "Use-Dilution" carrier methods were studied rather intensively, and a multiple metal-ring carrier method described by Stuart, Ortenzio, and Friedl (1953) was accepted as First Action by the Association of Official Agricultural Chemists for determining the maximum safe use-dilution for disinfectants offered for use on surfaces where prior cleaning cannot be relied upon to remove organic matter that might interfere with the action of the germicide or to decrease the bacterial load to a relatively low level as well as for confirming the reliability of phenol coefficient values obtained in the Association of Official Agricultural Chemists Phenol Coefficient Method-Official.

Credit must be given to Rideal and Walker (1903) as being the first workers to recognize the need for a standard precise method for comparing and evaluating disinfectants; and they proposed the first test procedure containing the fundamental control provision that the resistance of the test organism to a standardized pure chemical, phenol, be determined at the time the test is made and this result taken into consideration in the interpretation of the final result obtained. In addition to phenol controls of known strength, this method specified standards for media, the use of a specific test culture according to a given routine, and for such apparatus as the loop needle employed in making transfers. This method has been revised and modified many times in the 50-year period from 1903 to 1953, but it should be noted that all of the suggested revisions have retained the basic concept of employing the pure chemical, phenol, as the standard of reference. The most important revisions of the initial Rideal-Walker Method might be listed as those proposed by the Lancet Commission (1909), Anderson and McClintic (1912), a Committee of the American Public Health Association (1918), the original authors (1921), Brewer and Reddish (1929), Ruehle and Brewer (1931), the British Standard Institution (1934), and the Association of Official Agricultural Chemists (1950). The modifications suggested in all the revisions listed above were directed primarily toward the development of a procedure of improved precision and accuracy. As a consequence of these revisions, more work has been done in the evaluation and standardization of procedure detail and in correlating the end results with results obtained under conditions of actual application with the phenol coefficient procedure than with all other suggested procedures combined during this period. It is not surprising, therefore, that the primary bacteriological testing method employed in the evaluation of most disinfectants at the present time is the official A.O.A.C. phenol coefficient procedure. Since first proposed, phenol coefficient procedures have served as a nucleus around which storms of controversy have continually revolved. The propriety of almost every detail in the technique has been challenged repeatedly as well as the procedures of calculating and applying the results.

The method is actually a dilution tube procedure in which the highest dilutions of the germicidal chemical under investigation that kill the selected test organism within a series of time intervals under specified conditions are determined. From these results and comparable results secured at the same time with the pure chemical, phenol, a specific calculation is made to yield a product; namely, the phenol coefficient number. This number is then employed to calculate the dilution which might be presumed to be equivalent

in germicidal activity to a 5 per cent solution of the pure chemical, phenol, or as the maximum dilution that can be relied upon to disinfect under conditions commonly encountered in actual use. It should be emphasized here that no evidence has ever been found to suggest that the maximum killing dilutions found in the test can be relied upon to provide disinfection in usual recommended applications. These dilutions are used, therefore, solely for the calculation of the dilution presumed to be required for this result. As compared to the various carrier methods previously referred to, the phenol coefficient method must be classified as a method that yields a result which can only be interpreted indirectly in terms of the concentration of the product necessary for actual disinfection.

Disinfection always has as its objective the destruction of infectious agents known to be or suspected of being present. When the phenol coefficient test was first proposed, typhoid fever was the infectious disease causing the greatest number of fatalities in humans. Since the causative agent of this disease, *Salmonella typhosa*, was the dangerous infectious agent most likely to be the object of any disinfection procedure, it is not surprising that it was selected as the test organism of choice for use in the method. This test organism has been continuously used for 50 years as the test organism of choice and is employed in the present method for all initial evaluations.

Walters (1917), McCoy, Stimson, and Hesseltine (1917), Shippen and Griffin (1923), Reddish (1926), Philbrick (1930), Brewer and Ruehle (1931), Klarmann and Shternov (1936), and Ostrolenk and Brewer (1949) have all called attention to the limited value of test procedures inherent in the use of a single test organism. The adoption of *Micrococcus pyogenes* var. *aureus* as a secondary test organism in the F.D.A. method\* (1931) partially corrected for this deficiency and this organism is employed along with *S. typhosa* in the present official—A.O.A.C. procedure. Even so, it would not be safe to accept the results of this test as an index to the effectiveness of products in disinfecting against such resistant infectious agents as *Mycobacterium paratuberculosis*, *Mycobacterium tuberculosis*, *Clostridium tetani*, or *Bacillus anthracis*. While there are several other microorganisms of pathogenic and epidemiologic significance likely to be the object of disinfection processes which possess resistances to chemical germicides markedly different from *S. typhosa*, Reddish (1935), Varley and Reddish (1936), and Stuart,

\* Method developed by Lloyd P. Shippen, George F. Reddish, C. M. Brewer, and G. L. A. Ruehle and identified as the "Food and Drug Administration phenol coefficient" in U. S. Department of Agriculture Circular No. 198, (1931).



Ortenzio, and Friedl (1953) have shown that with many of the more common types of disinfectants, phenol coefficient numbers determined, using *S. typhosa* can usually be employed in the conventional calculation procedure to provide a solution with sufficient germicidal activity to disinfect under circumstances most frequently encountered. Also, the specific strain of this organism employed in the test has maintained remarkably constant characteristics in the hands of many different investigators over the years. It was shown by Ortenzio, Friedl, and Stuart (1949) to be capable of maintenance without undergoing change due to mutation and to possess fixed characteristics reflected in a constancy of resistance to germicidal chemicals. It seems probable, therefore, that this organism will continue to be the test organism of choice in initial evaluations for some time to come.

Although *M. pyogenes* var. *aureus* was recommended as a secondary test organism in the F.D.A. method (1931) and has been used extensively since that time, particularly to provide an index to the reliability of products for disinfecting in surgical wards, hospitals, clinics, and other places where pyogenic bacteria might be expected to have special significance, no specific instructions were given for the interpretation of the result obtained with this organism. Neither does the present A.O.A.C. Phenol Coefficient Method—Official definitely stipulate how the result obtained with this organism shall be interpreted. This oversight provided an opportunity for conflicting interpretations to develop, resulting in some confusion. The work of Stuart, Ortenzio, and Friedl (1953) shows very clearly that the maximum killing dilutions found in this method for this organism cannot be relied upon to disinfect where pyogenic bacteria are present, but that the phenol coefficient number found with this organism can usually be employed as a satisfactory index to a safe use-dilution for disinfecting against these organisms when multiplied by 20 to determine the number of parts of water in which one part of germicide should be incorporated. This organism was also found by Ortenzio, Friedl, and Stuart (1949) to be capable of maintenance at constant resistance levels to the chemical phenol, but experience indicates that it may be more susceptible to change due to mutation than *S. typhosa*.

Commercial germicides are expected to disinfect effectively in the absence of added heat. Exposure temperatures of 15° to 18°C were initially recommended in the Rideal-Walker Method. Anderson and McClintic (1912) adopted a temperature of 20°C for the Hygienic Laboratory Method, and this temperature has been accepted as the standard in all subsequent revisions of the phenol coefficient method. In the absence of ice or special equip-

ment, this is frequently found to be an inconvenient temperature to maintain in a laboratory water bath. The marked differences in the effects of increasing temperatures above 20°C on the various germicidal chemicals have ruled effectively against all suggestions that this temperature be increased.

Wright (1917) called attention to the importance of test culture medium composition in disinfectant testing, and Corper and Cohn (1938) have pointed out that the greatest single factor influencing the resistance of bacteria to chemical germicides is the chemical composition of the medium in which they are grown. Variations in results obtained in the phenol coefficient procedure have been traced on numerous occasions to variations in the beef extract, Goetchius (1950), and in the peptone, Brewer (1943), specified for use in preparing the standard medium employed in propagating the test culture. There is no doubt that more effective standardization of test culture medium ingredients would result in a marked improvement in the precision of the method. In this connection, it is of interest to note that beef extracts and peptone stipulated for use in preparing the media employed in all the various modifications of the test have always been identified according to a trade name, as—for example: "Liebig's," "Difco's," and "Armour's" without further description. Pelczar (1952) pointed out that inclusion of further identification of these ingredients in the method itself with respect to, (1) the nature of the raw materials from which they are derived, (2) the type of preparation employed, as—for example, acid hydrolysis, tryptic digestion, or peptic digestion, (3) chemical composition, and (4) bacteriological properties will be necessary before any marked permanent improvement can be anticipated on this score.

Various proportions of test culture volume to the volume of germicide to be tested have been proposed. Anderson and McClintic (1912) pointed out the necessity for accurate measurement of these volumes to assure the maintenance of a constant ratio if a precise result was to be obtained. As this ratio will also govern to some extent the end point obtained in the method with many products, any change therein over that specified in the recognized method would automatically have the effect of changing the commonly accepted standard for disinfecting unless, of course, a subsequent revision in the method for interpreting the laboratory result in terms of practical value was also made. In this connection, it might be pointed out that workers frequently increase the proportion of culture to germicide and add various amounts of organic matter to obtain a result which they can interpret directly in terms of presumed safe use-dilutions. This procedure has not been successfully standardized.

While the problem of transferring bacteriostatic quantities of disinfectants into the subculture medium is seldom as acute in the phenol coefficient procedure as in the various carrier methods previously referred to, it is occasionally a serious factor. The Shippen (1928) subtransfer method in which 4 loopfuls of the first subculture are transferred to a second tube of subculture medium provides an effective check on this factor with many types of germicides. However, the introduction in recent years of chemicals bacteriostatic even in excessively high dilution has led numerous investigators, Quisno, Gibby, and Foter (1946), Armbruster and Ridenour (1947), Tilley (1948), Klarmann and Wright (1948), Morton, North, and Engley (1948), to employ subculture media containing materials capable of suppressing bacteriostasis by the disinfectant being tested. Procedures of this type were accepted for use in the A.O.A.C. Phenol Coefficient Method—Official (1950).

The latitude allowed in determining the phenol coefficient number in the original Rideal-Walker Method constituted possibly its greatest weakness. Steps were taken to overcome this by Anderson and McClintic (1912), but the most effective correction of this deficiency came in the establishment of standards for the resistance of the test organisms to the control chemical, phenol, by Reddish (1926), (1927), and Ruehle and Brewer (1931).

The method depends upon the random sampling of the test organism-disinfectant mixture with a loop to determine the presence of surviving organisms at the end of the exposure time intervals prescribed. Cade and Halverson (1934) have discussed the factor of random sampling error as related to loop needle transfers of bacterial suspensions. While this error does not interfere greatly with the general accuracy of the phenol coefficient method with many products, it may become a matter of serious concern with chemicals which act to destroy a high percentage of the cells present in the test culture with extreme rapidity, but to kill the surviving cells at progressively diminishing rates of time. The difficulty of establishing an absolute end point in the method with products of this type has been pointed out by Stuart, Bogusky, and Friedl (1950).

Emmons (1933) described a phenol coefficient type procedure for measuring the activity of disinfectants against the spores of the pathogenic fungi *Trichophyton gypseum* and *Monilia albicans*. Klarmann, Shternov, and Costigan (1941) proposed a somewhat similar method employing a suspension of the spores of *Trichophyton rosaceum* as the test culture. Emmons (1944) pointed out that the test culture of *Trichophyton rosaceum* proposed by Klarmann, Shternov, and Costigan (1941) was in reality a saprophytic



*Fusarium* and could not be relied upon as an index organism for determining the activity of disinfectants against pathogenic fungi. A modification of Emmons (1933) original method using *Trichophyton interdigitale* was accepted as First Action—by the Association of Official Agricultural Chemists (1945). The method carries no specific instructions with respect to the interpretation of the result in terms of actual use. The most common interpretation given is that the maximum dilution killing in the method in 10 minutes is presumed to be the maximum dilution which could be expected to be effective in actual use. The alternative interpretation is that the product should not be employed where the object of the disinfecting process is a pathogenic fungus at a dilution greater than one part of product in a number of parts of water determined by multiplying the calculated phenol coefficient by 20. In the absence of actual use tests, this controversy cannot be accurately resolved.

Large quantities of germicidal chemicals are employed as an adjunct to cleaning operations with dishes, utensils, and cooking equipment in restaurants, with milking equipment on dairy farms, milk processing equipment in dairy plants, and with food-processing equipment in slaughter houses, egg breaking plants, creameries, bakeries, and the like as well as in breweries, wineries, and bottling plants. The objective here may be to disinfect or to destroy infectious agents suspected of being present or to obtain a general sanitizing effect in reducing the number of bacteria which will subsequently contaminate the foods or beverage to levels found necessary for avoiding spoilage.

The acceptable concentrations of the various chemicals employed in this field for disinfecting or sanitizing have been arrived at, for the most part, in tests made under conditions of actual use. No standard precise laboratory procedure has been accepted for common use in evaluating the efficiency of products for these uses. It has been repeatedly demonstrated that the effectiveness of all of these chemicals at the concentrations found practical and acceptable by the various Public Health Codes in disinfecting depends directly upon the efficiency of the precleaning operation in reducing the amount of organic matter present to a level below that which will interfere with the action of the germicide and in reducing the number of bacteria present to relatively low levels. Studies have also shown speed of action to be especially important in obtaining the result desired in these uses.

It is apparent, therefore, that the requirements for a laboratory test suitable for evaluating disinfectants for these purposes might be considerably at variance with the requirements for laboratory tests for disinfectants intended for other uses. Johns (1947), and

Weber and Black (1948) have proposed bacteriological methods for the examination of disinfectants for these particular uses to reflect the types of activity considered to be most desirable. Until some precise laboratory method is accepted for general use with these materials, considerable uncertainty and confusion as to requirements cannot be avoided. Should a satisfactory test be developed for use in this field, it would not necessarily hold that the results obtained could be interpreted directly in terms of practical disinfection in other uses where the requirements might be entirely different.

Public health officials seem to be in agreement that chemical germicides recommended to disinfect previously cleaned dishes in restaurants, dairy equipment, and food utensils should be employed at concentrations to provide a germicidal activity equivalent to that which would be furnished by an equal volume of a commonly accepted chlorine type germicide at a concentration providing 200 parts per million of available chlorine. They also seem to agree that solutions equivalent in germicidal activity to solutions of the same chlorine type germicide containing 100 ppm of available chlorine can be employed where chemical control facilities are available to prevent the germicidal activity of the solution in use from falling below that which would be equivalent to 50 ppm of available chlorine. Cantor and Shelanski (1951) outlined a procedure for testing germicides which is especially adapted for determining germicidal activity equivalent concentrations with chemicals active at high dilutions. This method is, therefore, useful in determining, in a presumptive manner at least, the concentration of chemical germicides necessary for these particular applications.

## METHODS

The methods selected for inclusion herein are those considered to be the most convenient and useful to the control laboratory. All but one have been shown to be suitable for use in referee work. The latter appears to offer distinct possibilities for development as a referee method and has been found especially convenient for testing where short exposure intervals are desired and capacity is an especially important consideration.

### THE ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS PHENOL COEFFICIENT METHOD — OFFICIAL

The A.O.A.C. Phenol Coefficient Method is applicable to testing disinfectants miscible with water which act against bacteria in a

manner somewhat comparable to phenol and which do not exert bacteriostatic effects that cannot be neutralized by one of the three subculture media specified.

## I. USING SALMONELLA TYPHOSA

### REAGENTS

(a) *Culture media*.—(1) Nutrient broth—Boil 5 g of beef ext. (Difco), 5 g of NaCl and 10 g of Armour peptone (quality specially prepared for disinfectant testing) in 1000 ml of H<sub>2</sub>O, 20 minutes, make to volume with H<sub>2</sub>O, and adjust to pH 6.8 (using colorimetric method, adjust broth to dark green color with bromothymol blue). Filter through paper, place 10 ml quantities in 20 x 150 mm bacteriological test tubes, plug with cotton, and sterilize at 15 lbs steam pressure 20 minutes. Use this broth for daily transfers of test cultures. (2) Nutrient agar—Dissolve 1.5 per cent Bacto agar (Difco) in nutrient broth and adjust to pH 7.2 to 7.4 (blue-green color with bromothymol blue), tube, plug with cotton, sterilize at 15 lbs steam pressure 20 minutes and slant. (3) Subculture media—Use (a), (b), or (c), whichever gives lowest result: (a) Nutrient broth described in (1); (b) Fluid thioglycollate medium U.S.P. XIII: Mix 0.75 g of L-cystine, 0.75 g of agar, 2.5 g of NaCl, 5.5 g of dextrose, 5.0 g of H<sub>2</sub>O soluble yeast extract and 15.0 g of pancreatic digest of casein with 1000 ml of H<sub>2</sub>O, heat to dissolve on H<sub>2</sub>O bath, add 0.5 g of Na thioglycollate or 0.3 g of thioglycollic acid, and adjust with N/1 NaOH to pH 7.0  $\pm$  0.1; reheat without boiling and filter through moistened filter paper; add 1.0 ml of freshly prepared 0.1 per cent Na resazurin solution; tube in 10 ml quantities in 20 x 150 mm bacteriological test tubes, plug with cotton, and sterilize at 15 lbs steam pressure 20 minutes; cool at once to 25°C and store at 20° to 30°C; (c) "Lethen broth": Dissolve 0.7 g of lecithin (azolectin) and 5.0 g of sorbitarn monooleate (Tween 80) in 400 ml of hot H<sub>2</sub>O and boil until clear; add 600 ml of soln. of 5.0 g of beef extract (Difco), 10.0 g of peptone (Armour), and 5.0 g of NaCl in H<sub>2</sub>O, and boil 10 minutes; adjust with N/1 NaOH and/or N/1 HCl to pH 7.0  $\pm$  0.2, and filter through coarse filter paper; tube in 10 ml quantities in 20 x 150 mm bacteriological test tubes, plug with cotton, and sterilize at 15 lbs steam pressure 20 minutes. With oxidizing products and products with toxic compounds containing certain heavy metals like Hg, (b) will usually give the lowest result. With products containing cationic surface active materials, (c) will usually give the lowest result.

(b) *Test Organism*.—Hopkins strain 26 of *S. typhosa* (Zopf) Weldin, F.D.A. A.T.C.C. No. 6539 (formerly called *Bac. typhosus*



and *Eberthella typhosa*), carry a stock culture on nutrient agar slant. Transfer once a month and incubate new stock transfer 2 days at  $37^{\circ}\text{C}$ , then store at room temperature. From the stock culture inoculate tube of nutrient broth and make at least 4 consecutive daily transfers (not over 30) in nutrient broth, incubating at  $37^{\circ}\text{C}$  before using culture for testing (if only one daily transfer has been

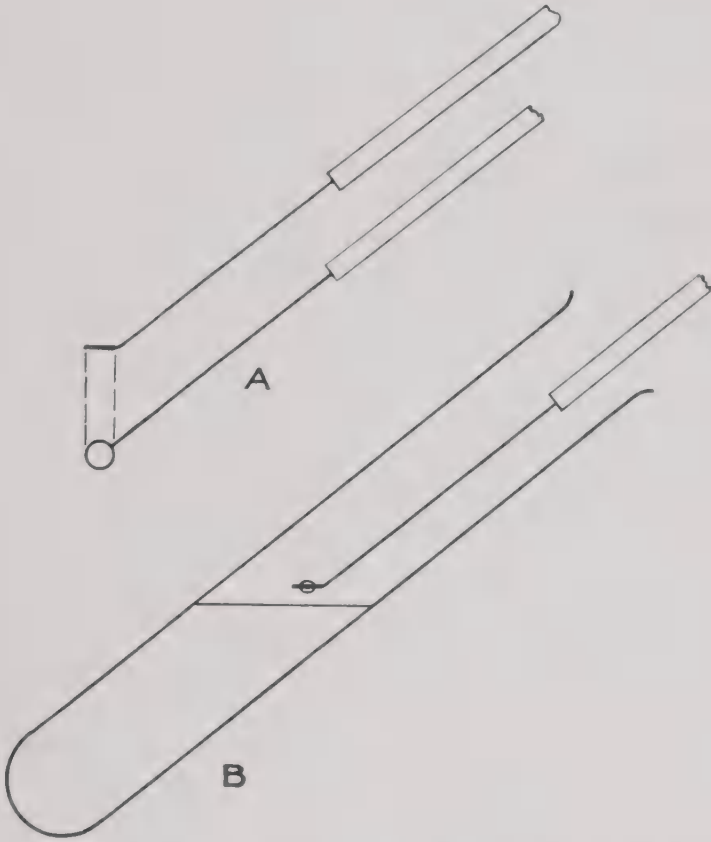


Fig. 1.—Transfer Loop and Manner of Using In Phenol Coefficient Method: A. Type of needle. B. Relation of loop to surface of liquid during transfer.

missed, it is not necessary to repeat the 4 consecutive transfers). Use 22 to 26 hours culture of organism grown in nutrient broth at  $37^{\circ}\text{C}$  in test. Shake, and allow to settle 15 minutes before using.

(c) *Phenol*.—Use phenol that meets requirements of U.S.P. and has congealing point of  $40^{\circ}\text{C}$  or above. Use 5 per cent solution as a stock solution and keep in well-stoppered amber bottles in relatively cool place, protected from light. Standardize with 0.1 N/K or Na bromide-bromate solution.

## APPARATUS

(a) *Glassware*.—1, 5, and 10 ml volumetric pipettes; 1, 5, and 10 ml Mohr pipettes graduated to 0.1 ml or less; 100 ml stoppered cylinders graduated in 1 ml divisions; pyrex lipped test tubes 25 x 150 mm, plug test tubes (medication tubes) with cotton wrapped in 1 layer of cheese cloth. Sterilize all glassware 2 hours in hot air oven at 180°C. Place pipettes in closed metal containers before sterilizing.

(b) *Water Bath*.—Insulated relatively deep H<sub>2</sub>O bath with cover having at least 10 well-spaced holes which admit medication tubes but not their lips.

(c) *Racks*.—May be of any convenient style. Blocks of wood (size depending somewhat on incubator to accommodate them) with deep holes are satisfactory. Have holes well spaced to insure quick manipulation of tubes. It is convenient to have them large enough to admit medication tubes while dilutions are being made.

(d) *Transfer Loop*.—Make 4 mm (inside diameter) single loop at end 2 to 3 inches Pt. or Pt. alloy wire No. 23 B&S gauge. Have other end in suitable holder (glass or Al. rod). Bend loop at 30° angle with stem (Figure 1).

## PROCEDURE

Make 1 per cent stock dilution of substance to be tested (or any other convenient dilution, depending on anticipated killing concentration) in glass stoppered cylinder. Make final dilutions, from the 1 per cent stock dilution, directly into medication tubes and remove all excess over 5 ml (range of dilutions should cover killing limits of disinfectant in 5 to 15 minutes and should at the same time be sufficiently close for accuracy). From the 5 per cent stock solution make 1 to 90 and 1 to 100 dilutions of the phenol directly into medication tubes. Place these tubes containing 5 ml each of final dilutions of disinfectant and phenol in H<sub>2</sub>O bath at 20°C and leave 5 minutes. Add 0.5 ml of the test culture to each of the dilutions at time intervals corresponding to intervals at which transfers are to be made. (Thus, by the time 10 tubes have been seeded at 30-second intervals, 4.5 minutes will have elapsed, and a 30-second interval intervenes before transference to subcultures is commenced). Add culture from graduated pipette of sufficient capacity to seed all tubes in any one set. As a precautionary measure loosely plug pipette with cotton at the mouth end before sterilizing it. The temperature of the culture should be practically that of the H<sub>2</sub>O bath before it is added.

In inoculating medication tubes, hold them in slanting position after removal from the bath, insert pipette to just above surface

of disinfectant, and run in culture without allowing tip to touch disinfectant. After adding culture, agitate tubes gently, but thoroughly, to insure even distribution of bacteria, and replace in bath; 5 minutes after seeding first medication tube, transfer 1 loopful of mixture of culture and diluted disinfectant from medication tube to corresponding subculture tube. To facilitate transfer of uniform drops of medication mixture, hold tube at 60° angle, and withdraw loop so that plane of loop is parallel with surface of liquid (Figure 1). At end of 30 seconds, transfer loopful from second medication tube to second subculture tube and continue process for each successive dilution; 5 minutes after making first transfer begin second set of transfers for 10-minute period, and finally repeat for 15-minute period. Gently agitate medication tubes before taking each interval loop subsample for transfer to subculture media. Before each transfer heat loop to redness in Bunsen flame and flame mouth of every tube. Sterilize loop immediately after each transfer (before replugging tubes) to allow time for cooling. Use care in transferring and seeding to prevent pipette or needle from touching sides or mouth of tubes; incubate subcultures at 37°C for 48 hours and read results. Thoroughly agitate individual subculture tubes before incubation. Macroscopic examination is usually sufficient, occasionally a 3-day incubation period, an agar streak, a microscopic examination, or agglutination with antityphoid serum may be necessary to determine feeble growth or suspected contamination.

#### CALCULATION

Express results in terms of phenol coefficient number or highest dilution killing the test organism in 10 minutes but not in 5 minutes, whichever most accurately reflects germicidal value of the disinfectant. The phenol coefficient is the number obtained by dividing the numeral value of the greatest dilution (denominator of the fraction expressing the dilution) of disinfectant capable of killing *S. typhosa* in 10 minutes, but not in 5 minutes, by the greatest dilution of phenol showing the same result.

#### EXAMPLE:

Disinfectant (x):	5 minutes	10 minutes	15 minutes
1-300	0	0	0
1-325	+	0	0
1-350	+	0	0
1-375	+	+	0
1-400	+	+	+
Phenol:			
1-90	+	0	0
1-100	+	+	+

$$\text{Phenol coefficient would be } \frac{350}{90} = 3.89 = 3.9$$



The test is satisfactory only when the phenol control gives one of the following readings:

Phenol:	5 minutes	10 minutes	15 minutes
1-90	+ or 0	+ or 0	0
1-100	+	+	+ or 0

If none of the dilutions of disinfectant shows growth in 5 minutes and killing in 10 minutes, estimate the hypothetical dilution only when any 3 consecutive dilutions show the following results; first, no growth in 5 minutes; second, growth in 5 and 10 minutes, but not in 15 minutes; and third growth in 5, 10, and 15 minutes.

EXAMPLE:

Disinfectant (x):	5 minutes	10 minutes	15 minutes
1-300	0	0	0
1-350	+	+	0
1-400	+	+	+
Phenol:			
1-90	0	0	0
1-100	+	+	0

$$\text{Phenol coefficient would be } \frac{325}{95} = 3.42 = 3.4$$

To avoid giving impression of false accuracy, calculate the phenol coefficient to the nearest 0.1. Thus, in the examples cited above the phenol coefficients would be reported as 3.9 and 3.4, instead of 3.89 and 3.42.

The commonly accepted criterion that disinfectants for general use be employed at a dilution equivalent in germicidal efficiency to 5 per cent phenol against *S. typhosa* (that is, 20 x *S. typhosa* coefficient) allows a reasonable margin of safety for destruction of infective agents likely to be the object of general disinfection. With products where it can be established that this does not hold true, such dilutions should not be used.

## 2. USING MICROCOCCUS PYOGENES VAR. AUREUS

Proceed as directed in using *S. typhosa*, except to change phenol dilutions and test organism. Use temperature of 20°C unless otherwise directed. Use culture of *M. pyogenes* var. *aureus* F.D.A., 209, A.T.C.C. No. 6538 (formerly known as *Staphylococcus aureus*), having at least resistance indicated by the following:

at 20° C.

<i>Phenol:</i>	<i>5 minutes</i>	<i>10 minutes</i>	<i>15 minutes</i>
1-60	+	0	0
1-70	+	+	+

Resistance of the culture to phenol used at 37° C. must be as follows:

<i>Phenol:</i>	<i>5 minutes</i>	<i>10 minutes</i>	<i>15 minutes</i>
1-80	+	0	0
1-90	+	+	+ or 0

## THE ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS USE-DILUTION CONFIRMATION TEST FOR RESULTS OBTAINED BY PHENOL COEFFICIENT PROCEDURE — FIRST ACTION

This method is applicable for use with water miscible disinfectants to confirm the validity of phenol coefficient data and/or to provide a direct measure of the safe use-dilution of germicides recommended for use on articles or surfaces where prior cleaning cannot be depended upon to eliminate interfering organic matter or reduce bacterial contamination to a relatively low level.

### 1. USING *SALMONELLA CHOLERAESUIS*

#### REAGENTS

(a) *Culture Media*.—The same as described in the A.O.A.C. Phenol Coefficient Method—Official, under 1.—Reagents (a) culture media.

(b) *Test Organism*.—*Salmonella choleraesuis*, A.T.C.C. 10708 carry a stock culture on nutrient agar slants. Transfer once a month and incubate new stock transfer 2 days at 37°C, then store at room temperature. From the stock culture inoculate tube of nutrient broth and incubate at 37°C. Make 3 consecutive 24 hour transfers, then inoculate tubes of nutrient broth (2 for each 10 carriers to be tested) using one loop of inoculum with each tube, incubate at 37°C for 44 to 48 hours and use in test.

(c) *Phenol*.—The same as described in the A.O.A.C. Phenol Coefficient Method—Official, under 1.—Reagents (c).

(d) *Sterile Distilled H<sub>2</sub>O*.—Prepare stock supply of dist. H<sub>2</sub>O in Erlenmeyer or Florence flasks, plug with cotton, sterilize at 15 lbs steam pressure for 20 minutes. Use to prepare dilution of medicants.

(e) *Asparagine*.—Make stock supply of 0.1 per cent solution of lacto-asparagine (Difco) in distilled H<sub>2</sub>O, in Erlenmeyer flasks of convenient size, plug with cotton, and sterilize at 15 lbs steam

pressure for 20 minutes. Use to cover metal ring carriers for sterilization and storage.

(f) *N/1 NaOH*.—Maintain stock supply of NaOH solution of approximately N/1 or 4 per cent concentration. Use for cleaning metal ring carriers prior to employment in the test.

## APPARATUS

(a) *Glassware*.—The same as described in the A.O.A.C. Phenol Coefficient Method—Official, under 1. Apparatus (a) except add a supply of 15 x 110 mm petri dishes sterilized in closed metal containers at 180° C for 2 hours and a supply of Erlenmeyer or Florence flasks of convenient volumes. Have 6 sterile petri dishes matted with a layer of No. 597 9cm filter papers. Use to drain and dry contaminated ring carriers.

(b) *Water Bath*.—The same as described in the A.O.A.C. Phenol Coefficient Method—Official, under 1. Apparatus (b).

(c) *Racks*.—The same as described in the A.O.A.C. Phenol Coefficient Method—Official, under 1. Apparatus (c).

(d) *Transfer Loop and Hook*.—The transfer loop shall be the same as described in the A.O.A.C. Phenol Coefficient Method—Official, under 1. Apparatus (d), in addition make a 3 mm right angle bend at the end of a 2 to 3 inch Nichrome wire needle of No. 18 B&S gauge. Have other end in suitable holder, (glass or aluminum).

(e) *Carriers*.—Polished stainless steel cylinders (penicillin cups)\* with an outside diameter of 8 mm ( $\pm 0.1$ ).\*\*

## PROCEDURE

Soak ring carriers overnight in a N/1 NaOH solution, rinse with tap water until rinse water gives a neutral reaction to phenol phthalein, then rinse 2 times in distilled water. Place cleaned ring carriers in numbers corresponding to multiples of 10 in cotton-plugged Erlenmeyer flasks or 25 x 150 mm cotton-plugged pyrex test tubes, cover with 0.1 per cent solution of Bacto-asparagine, sterilize at 15 lbs steam pressure for 20 minutes, cool and hold at room temperature. Transfer 20 sterile ring carriers, using a flamed nichrome wire hook, into 20 ml of a 44 to 48 hour nutrient broth test culture in a sterile 25 x 150 mm mediant tube. After a 15-minutes contact period, remove the cylinders, using a flamed nichrome wire hook, and place on end in a vertical position in a sterile petri dish matted with filter paper. Place in incubator at 37°C and

\* See specifications in Federal Register. 12, No. 67, p. 2217, April 4, 1947.

\*\* May be purchased from Ericksen Screw Machine Products Company, 25 Lafayette Street, Brooklyn, New York.



allow to dry for no less than 10 minutes and no more than 60 minutes. Hold the broth culture for determining its resistance to phenol according to the A.O.A.C. Phenol Coefficient Method—Official. From the 5 per cent stock solution make 1 to 80, 1 to 90 and 1 to 100 dilutions of the phenol directly into medicant tubes. Place these tubes containing 5 ml each in water bath and allow to come to temperature. Make stock solution of the germicide to be tested in sterile glass stoppered cylinder. From the solution make 10 ml volumes of the dilutions to be tested depending upon the phenol coefficient found and/or claimed against *S. typhosa* (1 ml of germicide in a number of ml of water determined by multiplying the phenol coefficient number by 20) directly into each of 10-25x150 mm medicant tubes, then place the 10 tubes in the H<sub>2</sub>O bath at 20°C and allow to come to temperature.

Determine the resistance of the test culture to the 1 to 80, 1 to 90 and 1 to 100 dilutions of phenol as outlined in the A.O.A.C. Phenol Coefficient Method—Official, under Procedure. Then add 1 contaminated dried cylinder carrier to each of the 10 tubes of the dilution of germicide to be checked allowing a 1-minute interval between each tube in the series. Thus, by the time the 10 tubes have been seeded, 9 minutes will have elapsed and a 1-minute interval intervenes before transferring the first carrier in the series to an individual tube of subculture broth, and this interval is a constant for each tube with the prescribed exposure period of 10 minutes. The 1-minute interval between transfers allows adequate time for flaming and cooling the nichrome wire transfer hook and making the transfer in a manner so as to drain all excess medicant from the carrier. Flame lips of the medicant and subculture tubes in the conventional manner.

Immediately after placing the carrier in the medicant tube, swirl the tube 3 times before placing it back into bath. Shake subculture tubes thoroughly, incubate at 37°C for 48 hours, and report results as + (growth) or — (no growth) values. Where there is reason to suspect that lack of growth at the conclusion of the incubation period may be due to the bacteriostatic action of the medicant adsorbed to the carrier which has not been neutralized by the subculture medium employed, each ring shall be transferred to a new tube of sterile medium and reincubated for a additional period of 48 hours at 37°C. Results showing no growth on all 10 ring carriers would be interpreted as confirming the phenol coefficient number found and/or claimed. Results showing growth on any of the 10 carriers should be interpreted as indicating the phenol coefficient number to be an unsafe guide to the dilution for use. In the latter case, the test should be repeated using lower dilutions of the germi-

cide under study. The maximum dilution of the germicide that kills the test organism on all 10 ring carriers in the 10-minute interval would represent the maximum safe use-dilution.

## 2. USING MICROCOCCUS PYOGENES VAR. AUREUS

Proceed as directed in 1. using *S. choleraesuis*, except to change the phenol dilutions and the test organism. Use culture of *M. pyogenes* var. *aureus* F.D.A. 209, A.T.C.C. No. 6538 having at least the resistance specified for the 24-hour culture at 20°C in the phenol coefficient method.

If the germicide does not kill the test organism on all 10 of the 10 carriers at the dilution indicated to be safe by the *S. typhosa* coefficient found and/or claimed, then it should not be recommended at this dilution for disinfecting in hospitals or places where pyogenic bacteria are likely to have special significance. If it does not kill the test organism on all 10 of the 10 carriers at the dilution indicated to be safe by the *M. pyogenes* var. *aureus* coefficient when this number is multiplied by 20 to determine the number of parts of water in which 1 part of product can be diluted, this dilution should not be recommended for disinfecting against pyogenic bacteria.

## CONSTANT BOILING HYDROCHLORIC ACID METHOD FOR DETERMINING SPOROCIDAL ACTIVITY OF CHEMICAL GERMICIDES AND THEIR POTENTIAL VALUE IN DISINFECTING WHEN THE OBJECT IS THE DESTRUCTION OF AN INFECTIOUS SPORE-FORMING ORGANISM

This method is applicable for use with water miscible disinfectants to determine the presence or absence of sporocidal activity and potential effectiveness in disinfecting against a specified spore forming bacteria.

(a) *Culture Media*.—(1). Soil extract nutrient broth.—Extract 1 pound of garden soil in 1 l of distilled water, filter several times through S&S No. 588 paper and bring to volume. The pH of this extract should be 5.2 or higher. Add 5 g of beef extract (Difco), 5 g of NaCl, and 10 g of Armour's peptone, boil for 20 minutes, adjust to pH 6.9, filter through paper, bring to volume and dispense in 20 x 150 mm test tubes, plug with cotton, and sterilize at 15 lbs steam pressure for 20 minutes. Use this broth for propagation of test culture of *Bacilli* (2). Nutrient Agar.—The same as described in the A.O.A.C. Phenol Coefficient Method—

Official, under 1. Reagents (a) (2). Use slants of this medium for maintaining stock culture of *Bacilli*. (3) Fluid Thioglycollate Medium U.S.P. XIII. The same as described in the A.O.A.C. Phenol Coefficient Method—Official, under 1. Reagents, (a) (3) (b), except to add 20 ml of N/1 NaOH to each liter prior to dispensing for sterilization. Use this medium for subculturing spores exposed to constant boiling HCl. For spores exposed to unknown germicides use Fluid Thioglycollate Medium U.S.P. XIII. (4) Soil extract meat egg medium—Add 1.5 g meat egg medium to individual 20 x 150 mm test tubes, then add 10 ml of garden soil extract as described above, plug with cotton, and sterilize at 15 lbs steam pressure for 20 minutes. Use this medium for propagating test cultures of *Clostridia* and maintaining stock cultures of species of this genus.

(b) *Test Organisms*.—Any species of *Clostridia* or *Bacilli*: Strains of *Bacillus subtilis* and *Clostridium sporogenes* may be employed in routine evaluations, but the method is applicable for use with strains of *B. anthracis*, *Cl. tetani* or other species in which the investigator may have an interest.

(c) *Constant Boiling HCl*.—Dilute analytical—reagent grade HCl (33 to 37 per cent HCl) with equal quantity of H<sub>2</sub>O. Adjust sp gr if necessary, to 1.10 (spindle). Place 1500 ml in 2 l flask connected to straight inner-tube condenser, using rubber or ground-glass stoppers. (Distilling system should be of resistant glass constructed so as completely to condense all distillate and at the same time maintain exact atmospheric pressure throughout). To prevent super heating of any part of the system, place ten 1" lengths of small diameter, resistant glass tubing in boiling flask and so encase flask in sheet asbestos that hot gases from flame strike flask only at bottom. Distill continuously at 5 to 10 ml/minute. When 1125 ml has been distilled, change receivers and catch next 225 ml which is constant boiling HCl in Erlenmeyer flask, with end of condenser inserted into flask but not below surface of liquid. Use this for determining resistance of the dried spores. The exact hydrochloric acid concentration may vary slightly depending on the atmospheric pressure. At 780 mm pressure, the concentration will be 20.173 per cent, and at 730 mm pressure, it will be 20.293 per cent or a mean molarity of approximately 5.5.

## APPARATUS

(a) *Glassware*.—Pyrex lipped test tubes 25 x 150 mm; 100 ml stoppered cylinders graduated in 1 ml divisions; supply of 15 x 110 ml petri dishes matted with 2 sheets of filter paper. Sterilize all glassware 2 hours in hot air oven at 180°C.



(b) *Water Bath*.—The same as specified in A.O.A.C. Phenol Coefficient Method—Official, under 1. Apparatus (b).

(c) *Racks*.—The same as specified in the A.O.A.C. Phenol Coefficient Method—Official, under 1. Apparatus (c).

(d) *Transfer Loop and Hook*.—The same as specified in the A.O.A.C. Use-Dilution Confirmation Test—First Action, under 1. Apparatus (d).

(e) *Suture Loop Carrier*.—From a spool of size 3 surgical silk suture, prepare standard loops by wrapping the silk around an ordinary pencil 3 times, slipping the coil so formed off the end of the pencil, and holding it firmly with the thumb and index finger while passing another piece of suture through the coil, knotting and tying it securely. Then shear the end of the coil and knotting suture off to within  $1/16$  of an inch. This should provide an overall length of approximately  $2\frac{1}{2}$  inches of suture in a two loop coil that can be conveniently handled in ordinary aseptic transfer procedures.

## PROCEDURE

Grow all *Bacilli* in soil extract nutrient broth and all *Clostridia* in soil extract meat egg medium. Inoculate tubes using 1 loop of the stock culture and incubate at  $37^{\circ}\text{C}$  for 72 hours. A supply of suture loop carriers are placed in petri dishes matted with filter paper and sterilized at 15 lbs for 20 minutes. New loops are used for each test. Five sterile loops are placed in each 72 hour culture, then the tube agitated vigorously, and allowed to stand for 15 minutes. Withdraw the loops and place in sterile petri dishes that have been matted with 2 sheets of filter paper, and allow to dry for 22 to 26 hours at room temperature. All suture loop transfers are made with a 2 to 3" nichrome wire hook. This is flamed in the conventional manner between transfers.

Transfer 10 ml of constant boiling hydrochloric acid into a sterile cotton plugged 25 x 150 mm lipped test tube, and then place the tube in a constant temperature water bath at  $20^{\circ}\text{C}$ , and allow it to come to temperature. Transfer 4 dried contaminated loop suture carriers to the tube of acid. Theoretically these transfers should be made simultaneously but practically a few seconds will elapse in making 4 successive transfers. Transfer the remaining dried contaminated suture loop to a tube of the thioglycollate subculture medium as a viability control. After 5, 10, 20, and 30 minutes individual loops are withdrawn from the acid and transferred to individual tubes of thioglycollate subculture medium. Rotate each tube vigorously for 20 seconds and incubate for 1 week at  $37^{\circ}\text{C}$ .

Reliable readings can usually be made after 48 hours incubation, but this will not always be true. If it appears that the pH of the subculture medium has been reduced by the acid carried over to a level below that which will permit growth of the test organism, transfer the loops to fresh tubes of media and reincubate for a second period of 7 days. The test spores should resist the HCl for at least 5 minutes. (Many will resist the HCl for the full 30-minute period and longer.) If this test shows that resistant dried spores are present, (vegetative cells will not show a measurable resistance against constant boiling HCl) duplicate lots of dried contaminated suture loops made with duplicate tubes of 72-hour broth cultures, in groups of 5, drained, and dried at the same time and held at room temperature for 7 days, are used for tests on the germicide to be investigated for sporocidal activity. (It has been established that spores dried and held under these conditions will retain their resistance for this period of time or longer.) Ten ml of the disinfectant at the dilution recommended for use or under investigation is placed in a 25 x 150 mm lipped test tube, and the tube placed in the water bath at 20°C and allowed to come to temperature. A set of 5 dried contaminated suture loops shown to be carrying resistant spores of the culture under study is selected and used for tests on each dilution of disinfectant. Transfer 1 suture loop immediately to a tube of thioglycollate subculture medium as a viability control. Then, transfer the remaining 4 loops to the dilution of disinfectant in the water bath. Remove individual loops at 4 selected intervals of time, as—for example: 10 minutes, 30 minutes, 1 hour, and 2 hours, transferring them to individual tubes of thioglycollate subculture medium. Shake all tubes thoroughly and incubate for 1 week at 37°C. If no growth occurs, and if there is reason to suspect that lack of growth may be due to bacteriostasis, transfer each loop to a fresh tube of medium and incubate for a second period of 7 days at 37°C. Report the results as + (growth) or — (no growth) values.

Dilutions of unknown germicides found to be effective against specific spores in this test might be expected to be effective in disinfecting against the same spores in actual use, providing an adequate contact period can be provided.

#### THE ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS FUNGICIDAL TEST — FIRST ACTION

This method is applicable for use with water miscible type fungicides used to disinfect inanimate articles and surfaces where the objective of the disinfection process is the destruction of pathogenic fungi infectious to man and animals.

*Using Trichophyton Interdigitale.*—Use as test fungus typical strain of *Trichophyton interdigitale* isolated from dermatophytosis of the foot, strain must sporulate freely in artificial media, presence of abundant conidia being manifested by powdery appearance on surface of 10-day culture, particularly at top of agar slant, and confirmed by microscopic examination. Conidia-bearing mycelium should peel easily from surface of dextrose agar. Conidia of required resistance survive 10-minute exposure at 20°C to phenol diln of 1:60\* but not of 1:45 (strain No. 640 American Type Culture Collection is suitable).

*Culture Medium.*—Carry fungus on agar slants of following composition: Dextrose 2 per cent, Neopeptone (Difco) 1 per cent, agar 2 per cent, adjusted to pH 5.6 to 5.8. Use same culture medium in preparing cultures for obtaining conidial suspension, and use fluid medium of same nutrient composition (without agar) to test viability of conidia after exposure to fungicide.

*Care of Fungus Strain.*—Store stock culture of fungus on dextrose agar slants at 2° to 5° at intervals not to exceed 3 months, transfer it to fresh agar slants, incubate at 25° to 30° 10 days, and then place in storage at 2° to 5° until next transfer period. Do not use culture that has been kept at room or higher temperatures for more than 10 days as source of inoculum for culture purposes. (Cultures may be kept at room temperatures for preservation of strain and for inoculation of cultures if transferred at intervals not exceeding 10 days.)

*Preparation of Conidial Suspension.*—Prepare petri dish agar cultures by planting inoculum at center of agar plate and by incubating culture at 25° to 30°C for 10, not exceeding 15 days. Then remove mycelial mat from agar surface of each plate by means of sterile spatula or heavy flattened wire and suspend in 20 to 25 ml of physiological NaCl (0.85 per cent NaCl). Free conidia from mycelium by shaking mixture with glass beads, filter through sterile absorbent cotton to remove hyphal elements, and dilute with physiological NaCl solution so that test suspension will contain 5 million conidia/ml. Estimate densities of conidia in suspension microscopically, using haemocytometer.

*Operating Technique.*—Prepare dilutions of the fungicide and employ similar procedure as outlined for the A.O.A.C. Phenol Coefficient Method—Official. Place 5 ml portions of each fungicide solution and the phenol control solutions in 25 x 150 mm test-culture tubes, arrange in order of ascending dilutions, and place tubes in

\* Experience now indicates that this dilution should possibly be increased to 1:65, but this change has not as yet been accepted by A.O.A.C.



20° H<sub>2</sub>O bath until temperature of bath is reached. With graduated pipette place 0.5 ml of spore suspension in first tube of fungicidal solution, shake, and immediately replace in H<sub>2</sub>O bath; 30 seconds later add 0.5 ml of the conidial suspension to second tube. Repeat procedure at 30-second intervals for each fungicidal dilution. If more convenient, run test at 20 second intervals. After 5-, 10-, and 15-minute periods of exposure to fungicide, remove sample from each conidia-fungicide mixture, with 4 mm loop, and place in 10 ml of the dextrose broth. To eliminate risk of faulty results owing to possibility of fungistatic action make subtransfers by withdrawing loopful of the conidia fungicide mixture, touching to surface in tube of dextrose broth as described above, and immediately immersing it in a second tube of broth. Incubate inoculated tubes at 25° to 30°C. Read final results after 10 days, although indicative readings can be made in 4 days.

### THE CANTOR-SHELANSKI CAPACITY TEST

This test is applicable to water miscible disinfectants and useful in determining germicidal equivalents, especially with products offered for use as germicidal rinses for previously cleaned surfaces or as drinking water disinfectants.

#### REAGENTS

The same as employed in the Association of Official Agricultural Chemists Phenol Coefficient Method—Official, and in addition, a supply of sterile distilled water, a supply of sterile M/15 KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> buffer pH 8.0, standard stock solution of NaOCl (approximately 5 per cent), and a standard stock solution (approximately 5.0 per cent) of KMnO<sub>4</sub>. Store stock solution of NaOCl in tightly closed bottle in icebox, and determine exact available chlorine concentration at frequent intervals by Arsenious Oxide Titration Method.\* Store stock solution of KMnO<sub>4</sub> in amber bottle protected from light at room temperature (above 15°C), adjust KMnO<sub>4</sub> solution to 50 g/l after dissolving 0.5 g Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub> in 300 ml of H<sub>2</sub>O and 10 ml of H<sub>2</sub>SO<sub>4</sub>, heating to 75° to 80°C and titrating with KMnO<sub>4</sub> solution:  $235.89 \div \text{result of titration in ml} = \text{concentration KMnO}_4 \text{ in g/l}$ . Use *S. typhosa* or *M. pyogenes* var. *aureus* or both as test organisms.

#### APPARATUS

The same as employed in the Official A.O.A.C. Phenol Coefficient Method.

\* Official Methods of Analysis A.O.A.C. 7th Ed., (1950), p. 80.

## PROCEDURE

1. *For Determining Available Chlorine Equivalent Concentrations with Germicides Offered to Disinfect Previously Cleaned Surfaces.*—Determine the resistance of the test culture to phenol at the dilutions and according to procedure outlined in the A.O.A.C. Phenol Coefficient Method—Official. Cultures having the resistance specified in that method should be employed. Using sterile M/15 phosphate buffer pH 8.0 and stock solution of NaOCl, make up solutions of NaOCl to contain 50, 100, and 200 ppm of available chlorine in sterile glass-stoppered cylinders. Transfer 5 ml of each solution to an individual 25 x 150 mm bacteriological test tube, and place the tubes for each concentration in water bath at 20°C, and allow to come to temperature. Starting with the tube containing 50 ppm of available chlorine, add 0.2 ml of test culture prepared as described in the A.O.A.C. phenol coefficient procedure, shake and return to water bath. After 1 minute, make a transfer to a tube of an appropriate subculture medium using a flamed 4 mm loop. At 1 minute and 30 seconds, add another 0.2 ml of culture, shake and return to bath. After an additional 1-minute interval (2.5 minutes in the test), make a second subculture in the same manner and within 30 seconds or at the 3-minute time in the test, add another 0.2 ml of culture, shaking and returning to the water bath. After another 1-minute interval (4.0 minutes in the test), make another transfer to a tube of subculture medium. Repeat this procedure to give a total of 10 added increments. This will require an over-all time for each solution of 14.5 minutes, and the addition of 2 ml of total culture with subculture at standard 1-minute intervals after addition of culture aliquots. At the conclusion of the test, shake all subculture tubes and incubate at 37° for 48 hours.

Repeat this procedure with the solutions containing 100 and 200 ppm of available chlorine. Make up solution of the unknown germicide at the concentration recommended or selected for study with sterile distilled water in glass-stoppered graduate. Transfer 5 ml to 25 x 150 mm bacteriological test tube, place in water bath and allow to come to temperature. Repeat the procedure with this solution.

To be considered equivalent in disinfecting activity to 50 ppm of available chlorine, the unknown germicide should show absence of growth in as many consecutive tubes of the subculture tube series as the 50 ppm available chlorine standard. Equivalent activity to 100 and 200 ppm of available chlorine would be determined in the same manner.

## EXAMPLE:

Germicide	Concn. ppm (av cl)	Subculture Series									
		1	2	3	4	5	6	7	8	9	10
NaOCl control	50	—	—	+	+	+	+	+	+	+	+
	100	—	—	—	+	+	+	+	+	+	+
	200	—	—	—	—	—	—	+	+	+	+
X	10	—	—	+	+	+	+	+	+	+	+
	20	—	—	—	—	+	+	+	+	+	+
	25	—	—	—	—	—	+	+	+	+	+

— = No growth

+ = Growth

From the above result, a 10 ppm solution of germicide X could be considered equivalent to a 50 ppm solution of available chlorine, and a 20 ppm solution equivalent to 100 ppm of available chlorine, but a 25 ppm solution of germicide X would not be considered as equivalent in germicidal activity to 200 ppm of available chlorine.

2. *For Determining Equivalent  $\text{KMnO}_4$  Disinfecting Values for Poultry Drinking Water.*—From stock solution, make up a 1 to 3000 dilution of  $\text{KMnO}_4^*$  in 100 ml sterile graduate employing sterile distilled  $\text{H}_2\text{O}$ . Transfer 5 ml of this solution to a 25 x 150 mm bacteriological test tube and allow to come to temperature. Proceed as under 1, except to reduce the volume of each increment to 0.1 ml and increase each exposure interval to 2 minutes. The over-all time for each solution will thus be increased to 24.5 minutes with a total addition of 1.0 ml of culture. Then transfer 5 ml of the solution of unknown germicide recommended or to be tested for this use to a similar test tube and proceed in the same manner. The test culture should have the resistance to phenol specified in the A.O.A.C. phenol coefficient procedure. Interpret result as to equivalent germicidal activity to a 1 to 3000 dilution of  $\text{KMnO}_4$  in the same manner specified under 1 for equivalent activities to NaOCl solutions. With a standard 24-hour culture of *S. typhosa* usually the first 3 tubes will be negative and all subsequent tubes in the series positive.

## COMMENT

Results in the test are useful in making estimates of equivalent efficiencies in uses of the type indicated. However, collaborative studies appear necessary especially in arriving at minimum and maximum time intervals before the method can be accepted for referee work.

\* Solution most commonly accepted for this use, see Tilley (1947).



## GENERAL CONSIDERATIONS

It should be noted that applicability in all of the 5 methods listed is linked to water miscibility. Bacteria are normally water-borne, and anhydrous preparations which are not soluble or miscible with water cannot, as a general rule, be relied upon to disinfect. It will be noted also that all 5 methods are complete end point methods which require, within the limits of experimental error, a 100 per cent kill of the test organism. In this connection, it should be pointed out that when it can be shown that the infectious agent, against which the disinfecting process is directed, is not capable of growth and reproduction or of initiating an infection, then and only then can disinfection be claimed to have been achieved.

Where the organism or infectious agent is such that it will not grow readily in artificial culture media as with *M. tuberculosis* and viruses, special methods have to be devised to provide an adequate and uniform supply of infectious material or organisms in the form in which it might normally be encountered for exposure to the disinfectant solutions, and subculture then made by what has been described by Dennis (1951) as an "*in vivo* challenge method." In a method of this type, aliquots of the medicant solution-infectious agent mixture are injected into a susceptible animal at the conclusion of each exposure interval.

With *M. tuberculosis*, heavy suspensions of organisms grown on artificial media can usually be employed with exposure in the procedure prescribed in the A.O.A.C. phenol coefficient method. The guinea pig is the susceptible animal of choice and inoculation of the control organism, (0.5 ml of suspension to 5 ml of physiological saline) and of the organism-medicant mixture is usually made intraperitoneally at the rate of 0.5 ml per 600 gm of body weight.

With infectious viruses, the problem is much more difficult especially with respect to selection of the infectious material suitable for exposure to the disinfectant solution to be tested. The procedures employed in this field of testing have been so variable in the past that it is very difficult to arrive at reliable estimates of practical value from the results published in the literature. McCulloch (1945) reported that emulsions of virus containing material such as the dried scabs of fowl pox could be employed in lieu of test cultures in a procedure similar to that employed in phenol coefficient testing with subculture in the embryo of the chick to provide useful data. The development of the technique of propagating viruses in the chicken embryo has opened up the possibility of developing a standard procedure employing emulsions of mascerated infected tissues and/or infected allantoic fluid for mixing with

dilutions of the germicide recommended followed by exposure at 20°C for varying periods of time in a procedure similar to the A.O.A.C. phenol coefficient method. Subculture at the various exposure time intervals can then be made from the medicant-infectious material mixture into embryos of suitable age.

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## 6

# FUNGISTATIC AND FUNGICIDAL TEST METHODS

## GENERAL CONSIDERATIONS

THE need for a standard test method for the evaluation of antifungal compounds used in the treatment of fungus infections of man and other animals has long been felt. Many methods, both *in vitro* and *in vivo*, have been suggested; but, because of various innate shortcomings, not one has as yet been generally accepted (Weidman *et al.*, 1945). In order to have a meaningful test, the method should be performed under laboratory conditions simulating the pathological situation as closely as possible. Drugs designed for the treatment of systemic mycoses, as histoplasmosis, actinomycosis, or coccidioidomycosis, may best be evaluated by *in vivo* testing, *i.e.*, animal experimentation. *Histoplasma capsulatum*, *Novcardia asteroides*, and *Coccidioides immitis* may be inoculated into animals by the suspension of the pathogen in 5 per cent gastric mucin. The therapeutic index of any proposed antifungal substance may then be determined by any standard method of chemotherapeutic testing (Campbell and Saslaw, 1950; Strauss and Kligman, 1951).

This chapter will deal, however, with organisms causing superficial mycoses, as *Microsporum*, *Trichophyton* and *Epidermophyton*, which do not lend themselves easily to *in vivo* experimentation. Animals seem to overcome experimentally produced infections spontaneously, and skin manifestations of the superficial mycoses may be evinced only by fairly artificial means, such as prolonged rubbing or pretreatment with certain hormones or castration. These methods are also not adapted easily to the screening of compounds or to mass testing (Reiss, 1947; Wharton, Reiss, and Wharton, 1950).

Of the superficial mycoses, fungus infection of the hair and nails should not be included *a priori* in the spectrum of any antifungal substance tested for dermatophytosis. The problem of penetration through the horny layers of the nails and the keratin of the hair is added to the demand for antifungal potency of any effective compound. Therefore, laboratory tests performed for the evaluation of chemicals on organisms invading the superficial skin do not yield any information about their eventual efficacy in tinea capitis caused by *Microsporum audouini* or *Microsporum lanosum*, even if these two fungi or their spores were included in the *in vitro* evaluation (Robinson, 1948).

In human or animal pathology the problems of treating superficial mycoses are: (1) eradication of fungi which are present in various stages of growth; (2) penetration of the chemical to the fungus through layers of skin containing proteins, phospholipids, and other substances; (3) prevention of harm to the skin by the substance and prevention of excessive general absorption through the skin; (4) avoidance of frequent sensitization; and (5) discovery of a potent substance which will be effective in a relatively low concentration and in a fairly brief contact time with the pathologic organism.

Considering these five points in succession; first, the test methods employed for human fungus infection should deal with a culture already in full growth, with all the growth elements present, such as hyphae and micro- and macroconidia. On the other hand, there exist the tests mainly designed for problems of plant pathology, where the main effort is placed on the prevention of fungus infections rather than the alleviation of existing ones. Here the toxicant is usually present before the fungus invasion. Growth of pathogenic fungi is merely inhibited, but only seldom treated (Horsfall, 1945). Therefore, tests designed to study fungicides under adverse growth conditions for the fungus will inevitably produce false information in human therapeutics, since unreasonably large dilutions of relatively weak and inefficient antifungal substances may, by these methods, produce antifungal effects which are meaningless in the therapy of human fungus diseases. Similarly, a multitude of compounds designed for human therapy have been tested in poor growth media, as broth or watery solutions, and have been wrongfully designated as effective fungicides.

Second, each antifungal substance should be tested in the presence of proteins and phospholipids. These substances should be incorporated in the growth medium, and their effect should be noted. Many halogen containing compounds and surface active agents would, under this scrutiny, lose much of their "laboratory" efficacy (Baker, 1941).



Third and fourth, after a substance has been shown through meaningful laboratory tests to possess good antifungal qualities, suitable *in vivo* tests should be performed on a sufficient number of animals and persons to rule out excessive keratolysis, excessive penetration into the underlying organs and general toxic effects, and also to rule out primary irritation or skin sensitization.

Fifth, with the advent of potent antibiotic chemotherapeutics the treatment time of certain diseases has been enormously shortened. For instance, the period of antiluetic therapy for tertiary syphilis has been shortened from about 11½ years to roughly 10 days to 2 weeks with penicillin therapy. Such a disease as tinea capitis, however, still requires about 4 to 9 months of daily treatment despite its relatively easy accessibility. Therefore, reasonable doubt is expressed about the validity of the claim for successful antifungal testing of many substances designed for the treatment of this condition. Many antifungal substances are used clinically in exceedingly high concentrations which are out of step with their tested *in vitro* effectiveness.

The antifungal tests should, therefore, offer an expression of the activity of the compound with decreasing dilution. A dosage response curve should be established to discover the concentrations at which the maximum activity has been attained. In fungicidal tests a reasonably short contact time between chemical and fungus is desirable in order to avoid unrealistically long periods of treatment. Furthermore, a test method should be relatively easy to perform, not requiring excessive technical skill or complicated apparatus. It should lend itself to routine testing and screening of compounds. The test time should be reasonably short (Oster and Golden, 1949).

### TERMINOLOGY

Before delving into the actual descriptions of the fungistatic and fungicidal test methods, it is important to define the meaning of the terms. Fungistatic action of a substance indicates its growth preventing effect on a fungus culture as long as it is in contact with the organisms. Fungicidal action, on the other hand, designates the killing effect of a compound on a fungus culture after contact for a limited period of time, making certain that the compound is completely removed from the culture following the contact time and that the basic structure of the colony has not been altered either by the compound or by the test method.

If one makes these definitions the criteria for any suggested test method, he would exclude, as far as *in vitro* test methods are concerned, all compounds which form permanent chemical com-

plexes with the fungus proteins, such as various mineral salts, and those which destroy the fungus itself, such as strong acids or bases. Likewise, such fungicidal methods which in their performance might alter the basic structure of the colony would be excluded by this definition (Golden and Oster, 1947).

## FUNGISTATIC TEST METHODS

There are two general methods for estimating the fungistatic effectiveness of chemicals, the Serial Dilution method and the Agar Cup Plate method; the former being first suggested by Schamberg and Kolmer (1922). A modification of the original method is here-with given. The accurately diluted test substance in aqueous solution, if possible, or in 0.1 ml of 95 per cent alcohol, is incorporated in plates containing 20 ml of Sabouraud's agar and at least 10 per cent horse serum. The freshly isolated fungus is seeded on the plate and incubated at room temperature. A number of dilutions of the drug should be used, and each test should be repeated to insure accuracy. After 2 weeks the presence or absence of growth is noted. The highest dilution of the test material which completely inhibits the growth of fungi after a 2-week incubation period is regarded as the critical fungistatic dilution.

This fungistatic test method is very valuable in the testing of volatile compounds. Its shortcomings are that the test reveals but one concentration value at which the substance is either fungistatic or not, and that nothing is learned of its essential activity or progressing and leveling off to a constant ceiling of fungistasis.

This particular information may be gained by another fungistatic *in vitro* test method, the so-called Agar Cup Plate method, originally introduced by Reddish in 1929 and described in the United States Department of Agriculture Circular No. 198 for the determination of bacteriostasis, and adapted for fungistatic testing by Burlingame and Reddish in 1939. Here, the serum agar is prepared by adding 2 ml of sterile normal horse serum to 18 ml of Sabouraud's agar, which has been melted and cooled at 40°C. The agar is poured into a 9 cm Petri dish, and, when hardened, the entire surface of each plate is streaked with a 10-day-old culture of the test organism. A 2 cm cup is cut from the center of the plate with a sterile cork borer, and approximately 0.8 ml of the test substance dissolved in 95 per cent alcohol or another suitable solvent is pipetted into the cup. The fungistatic activity of a given compound is indicated by a cleared zone around the cup after a growth period of 5 days at room temperature. The clearance is expressed as a radius segment in mm from the edge of the cup to

the beginning of growth by an average of at least 5 determinations. By measuring several clearances at different dosages, a slope of activity may be obtained using the dosage response curve.

From the dosage response curve an activity coefficient of a fungistatic substance may be calculated. As a standard for comparison, a 5 mm zone at 1 per cent concentration of the compound tested is arbitrarily chosen as the activity 1. Using *Trichophyton mentagrophytes* (640 Emmons) as the test organism, the concentration at which a compound exhibits 5 mm clearance divided into the figure 1 gives the fungistatic activity coefficient of this particular substance (Oster and Golden, 1947).

This method avoids reference to a standard fungistatic compound and refers only to a given area of radius segment clearance with a given organism. It is easily reproducible. A means of expressing fungistasis by measuring the activity coefficient derived from dosage response curves is provided. The standard conditions are those of medium and of culture. Fungistatic ointments and powders may be tested by but slight variation of the original method. Modifications of this method, such as the use of Penicups or filter paper disks, introduce inaccuracies and should not be considered for routine testing (Hillegas and Camp, 1945; Kligman and Rosensweig, 1948).

In recent investigations another old fungistatic method has been utilized which lends itself in a certain degree to the estimation of dosage response (Falck, 1907). Various dilutions of a fungistatic substance are incorporated into an agar plate. Into the center of the plate is placed a plug, 1 cm in diameter, of a 10-day-old fungus culture. At room temperature, the thallus of *T. mentagrophytes* requires approximately 18 to 21 days to cover the entire surface of the plate. With increasing concentrations of the antifungal substance lesser areas of the agar plate are covered with growth. A dosage response curve may be obtained by measuring the number of days required to reach a particular diameter or by the diameter reached in a specified number of days (Grunberg *et al.*, 1950).

This method omits the gradient of penetration through agar which enters in the Agar Cup Plate method. However, the increased time element of growth for the fungus culture in a poisoned medium may give rise to artificial growth elements or omission of certain growth stages of the fungus. Again, the pathological condition of the human skin existing in dermatophytosis is not paralleled by the test. Growth extends into a nutrient already possessing a toxicant and not vice versa, as it should be.

Following an idea of the plant pathologist of antidoting the toxins of fungi as plant disease control (Horsfall and Zentmyer,



1942), Peck (1949) attempted to base a new method for the evaluation of antifungal compounds on their effects on the metabolism of the fungi, as far as the formation of trichophytin is concerned. This method may be helpful for the study of the mechanism of action of the allergenic substances produced by fungi, but it does not contribute essentially to the measurement of fungistatic potency. It has not yet been proven, by any means, that the allergenic substances released by the fungus on the skin are identical with those originating from a partial suppression of fungus growth or those released by the sudden destruction of fungi by an actual fungicide.

### FUNGICIDAL TEST METHODS

Contrary to many antibacterial compounds which must exert only bacteriostasis to be clinically effective, it appears in the present stage of our knowledge that the superficial mycoses require true fungicidal agents for the same purpose. Fungistatic agents seem to give rise to frequent recurrences and to drive the active infection into a quiescent stage which might be reactivated whenever optimal growth conditions again exist. For this reason many tests designed to evaluate the fungicidal power of any substance have been devised.

The Council on Pharmacy and Chemistry of the American Medical Association (Weidman *et al.*, 1945) recommends an *in vitro* test adapting the phenol coefficient test for disinfectants and antiseptics as modified by the American Public Health Association subcommittee: (a) "The test fungus should be *Trichophyton interdigitale*. A suitable strain, No. 9533, is procurable from the American Type Culture Collection, Georgetown University, 3900 Reservoir Road, Washington, D. C. It should survive 10 minutes' exposure at 20° C to phenol 1 : 60 but not to a strength of 1 : 45." (b) "Spore suspensions of this test fungus should be prepared from 10-day agar cultures in a concentration of 5 million conidia per ml. For performing the test, 0.5 ml of this suspension is added to 5 ml of the fungicide concentration being tested." (c) "Samples for viability tests should be taken at 5, 10 and 15 minute intervals and planted in a liquid medium containing 1 per cent Difco Neopeptone and 2 per cent chemically pure Dextrose, pH 5.6 to 5.8. A liquid medium is essential for the rapid dissipation of the fungicide carried over. In the case of fungicides exerting a strong fungistatic effect, subcultures must be made." "This test is frankly an *in vitro* test such as is appropriate in testing the action of fungicides on floors and the like, and attempts to read more into it are ill advised."

The fungicidal method suggested by Schamberg and Kolmer (1922), repeated by McCrea (1931) and by Klarmann, Shternov,

and Gates (1934), tests spore suspensions also by breaking up the mycelial mat of the fungus by artificial means. However, it does not appear logical to test fungi like bacteria, disregarding the fact that the growth of a fungus culture occurs by hyphae matted together into a mycelium, plus a variety of spores, conidia, and chlamydospores. This objection was overcome by the fungicidal method developed by Burlingame and Reddish (1939), adapting a previously described method by Smyth and Smyth (1932).

Petri dishes of Sabouraud's agar are inoculated with a culture of the test organism and incubated at room temperature for 5 days, at which time the cultures are cut into 1 cm squares or disks by means of a cork borer 1 cm in diameter. The fungicide to be tested is poured over the surface of the cultures so as to entirely flood the plates. This requires 15 ml or more for each plate. After 5, 15, and 30 minutes one of the squares or disks of culture and agar is removed from each plate and placed into 10 ml of sterile broth. The excess fungicide is washed out of the matted culture by shaking the broth tube lightly for 5 minutes. At the end of this time the block of culture is removed from the broth and spread, culture side down, over the surface of a sterile slant of Sabouraud's agar. These slants are then incubated at room temperature for 3 weeks and observed for growth. An effective fungicide should kill the test organisms within 5 minutes.

This test is simple. It uses as test organisms, among others, *T. mentagrophytes*. The solid medium used is more adapted to optimal growth conditions of the fungi than liquid media. The washing step avoids a continued fungistatic effect which might still be present when loops of spore culture are transferred in other fungicidal tests.

The test was modified for water insoluble fungicides by Golden and Oster (1947). At the same time the criteria for fungicidal activity were made more severe. The age of the fungus test culture was raised to 15 days, serum was added to the culture medium, and a 1-minute contact time was followed by an acetone-water mixture washing step. This modified test is specified for alcohol soluble fungicides. Petri dishes of Sabouraud's agar containing 10 per cent horse serum are streaked with a culture of *T. mentagrophytes* (640 Emmons) and permitted to grow at  $28^{\circ} \text{C} \pm 1^{\circ}$  for 15 days. On the day of the test the cultures are cut into disks of 1 cm diameter with a sterile cork borer and transferred with aseptic precautions to seeding tubes containing 10 ml of the various concentrations of the test fungicides in 95 per cent ethyl alcohol. After a 1-minute contact with the fungicide, the disk is transferred to 10 ml of sterile

broth and shaken lightly for 3 minutes to free the matted culture of any water soluble or miscible material. The culture block is then removed from the broth and washed in 10 ml of 30 per cent acetone-water mixture for 5 minutes, thereby removing any fungicide adhering to the mycelia. Following this acetone washing step, the disk is once more immersed in sterile broth for 2 minutes to remove possible traces of acetone and then spread, culture side down, over the surface of a sterile slant of Sabouraud's agar. These final slants are incubated at  $28^{\circ}\text{C} \pm 1^{\circ}$  for 3 weeks and observed for growth.

This test method was also adopted for the evaluation of fungicidal ointments by the addition of nonionic emulsifying agents to the acetone-water washing solution, facilitating the removal of any adhering ointment from the culture disk (Golden and Oster, 1950).

## TEST OF THE ACTUAL ANTIFUNGAL PREPARATION

The test methods so far described deal with the evaluation of pure chemicals. In actual dermatological practice, however, the active principle is usually incorporated in some sort of vehicle or carrier. These substances, may they be ointments, waxes, solutions or powders, will exert an influence on the antifungal performance of the active principle. They may be completely inert, synergistic, or antagonistic. For this reason it should not be assumed that any preparation, because it contains a proven antifungal compound, will exert the same fungistatic or fungicidal influence as its active principle did when tested in water or another solvent. It is of the utmost importance to submit the final preparation to an appropriate test, since the affinity of the inert ingredients added to the active principle may in some instances be so great that almost none of it is available for release on the skin. Also it would not be good practice to test these preparations by extracting the active principle with a suitable solvent and then to claim the potency of the preparation by the amount which may be recovered. The compounding of the antifungal preparation must take into consideration the necessity for the gradient of concentration of the active ingredient to move from the vehicle toward the skin. This quality may be discovered by suitable fungicidal testing but not by the available fungistatic test methods, because any antifungal preparation worthy of its name should at least clear the entire test plate, if tested by the Agar Cup Plate method, in the concentrations to be used for therapy. For fungicidal testing a contact time of 2 minutes in the respective carrier instead of the 1-minute contact with 95 per cent alcohol should be sufficient. This test may also be helpful in the determination of the optimal concentration of the active fungicidal principle in the finished preparation.



Caution is recommended in the testing of finished preparations containing surface active chemicals, which may in some instances favorably influence the *in vitro* testing by causing increased penetration of the active ingredient. However, the values obtained may have only spurious significance. The preparation is compounded only testwise. To avoid pitfalls of this kind suitable inhibitory agents, such as phospholipids, should be added to the agar medium before definite statements of the *in vitro* activity of preparations containing wetting agents can be made. The final approval for any preparation, however, is afforded by clinical testing.

### CLINICAL TESTS

The Council on Pharmacy and Chemistry of the American Medical Association has set up 12 rules for the clinical testing of antifungal compounds (Weidman *et al.*, 1945). The observation of these can be largely recommended. The 12 points deal with (a) patient material should be properly selected and graded; (b) proper environmental factors among the test groups should be maintained; (c) clinical diagnosis should be confirmed by eventual laboratory evidence; (d) frequency and duration of treatment should be uniform; (e) the faithfulness of the patient to the treatment should be evaluated; (f) objectivity to the treatment results on the part of the patient should be established; (g) local irritant effects of a test fungicide should be substantially non-existent; (h) sensitivity to the fungicide should be watched for; (i) toxic systemic effects should be negligible; (j) reading of results of treatment should be done in the most unbiased manner; (k) a possible mycological check on the treatment results should be performed whenever possible; (l) the grading of the results should be uniform and follow an accepted pattern.

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7

## METHODS OF TESTING CHEMICAL STERILIZERS AND TESTS FOR STERILITY

THERE is no standard method for testing sporicidal activity of chemical sterilizers, and in fact no particular procedure that is generally used for the purpose. Different test methods have been employed for over half a century with the result that widely varying data have been reported. Among the factors responsible for these variations are differences in resistance of spores of various species of sporulating microorganisms, varying degrees of difference in spore resistance due to culture media employed, widely different test conditions, the use of a variety of subculture media, the presence or absence of bacteriostasis in subcultures, and certain other details in test procedures.

Regardless of the method employed, the high resistance of bacterial spores has been extensively established. There are many instances of bacterial spores surviving alcohol preservatives in museum pathological specimens for many years. Anthrax spores have been known to retain their virulence for 40 years when stored in dry gauze bandage (McCulloch, 1945), and many other instances of resistance to drying and heat have been reported. Laboratory studies have also demonstrated similar resistance under a variety of conditions.

The principal difficulties in the laboratory testing of chemical sterilizers involve the use of different sporulating organisms, use of a variety of culture media, and whether or not spore cultures were heated before use in sporicidal tests. For example, Hornung (1935), using anthrax spores, found that undiluted Lysol required from 34 to 48 hours to kill these spores, whereas Morton (1950)



reported that 50 per cent alcohol killed such spores within 48 hours. Since Lysol is many times more germicidal than 50 per cent alcohol, this difference in sporicidal activity must be due to differences in resistance of the spores used in the tests. Although not always specifically stated, spore suspensions are often heated to kill vegetative cells before the culture is submitted to sporicidal tests and this will affect spore resistance to germicides.

Reddish (1950) made a study of this factor and found that heating cultures of sporulating organisms to kill vegetative cells actually weakened the spores so that they were more easily killed by chemical sterilizers. Two representative aerobic spore-formers were used, namely *Bacillus cereus* and *Bacillus anthracis*, and tests were conducted with cultures before and after heating using four germicidal solutions. The germicides used were 70 per cent alcohol, 5 per cent phenol, 2 per cent tincture of iodine (U.S.P.), and 7 per cent tincture of iodine (N.F.) The cultures were grown in broth and on agar slants of the following composition: 0.5 per cent Difco beef extract, 1.0 per cent Armour peptone, 0.5 per cent NaCl, adjusted to pH 7.4. Broth of the same composition and fluid thioglycollate medium were each used as subculture media. The broth and agar slant cultures were grown at 37° C for 1 to 7 days, the agar slant culture being suspended in 10 ml of sterile saline before use. Both cultures were used in the sporicidal tests before and after heating at 80° C for 15 minutes. Five-tenths ml of each test culture was added to 5.0 ml of germicide at 25° C and loop transfers of the medication mixture made to 10 ml broth and 30 ml of fluid thioglycollate at 1 to 7 days and incubated at 37° C for 4 days.

The results of these tests may be briefly summarized. The heated and unheated spores of *B. cereus* and *B. anthracis* were not killed by 70 per cent alcohol after 7 days contact at 25° C and there was no apparent difference between cultures grown in broth and on agar slants. The results with 5 per cent phenol showed a significant difference in resistance of heated and unheated spores. It required from 3 to 5 days for 5 per cent phenol to kill the unheated spores of *B. cereus*, whereas the heated spores were killed within 1 to 3 hours. The unheated *B. anthracis* spores were killed within 3 days but not 2 days, whereas the heated spores were killed in 2 days but not in 1 day. It is apparent that the spores of both organisms were weakened by heating at 80° C for 15 minutes and that the effect of heating was greater in one species than in the other. The unheated spores of *B. cereus* were killed by 2 and 7 per cent tincture of iodine in 15 to 30 minutes, according to the age of the culture, whereas the heated spores were killed in from 5 to 15 minutes, according to the age of the culture.

These differences in resistance of unheated and heated spores may account for some of the variations of sporicidal tests reported. On the basis of these results it is apparent that only unheated spores should be used in sporicidal tests. This is important as a practical matter since spores are not preheated when germicides are employed under conditions of use. While the test method described may be used as a screening test the results cannot be interpreted in terms of practical value.

There is a need for a practical performance test for sporicidal activity, one that can be interpreted directly in terms of value under use conditions. Two such tests have been suggested, one by Reddish and Burlingame (1938) and one by Spaulding (1939). The two methods have much in common, but differ in one essential respect. The method suggested by Reddish and Burlingame is based on the premise that surgical instruments are cleaned before sterilizing, and in fact this is the customary procedure as regularly practiced. Although Spaulding recognized this, the method he suggested makes use of blood and pus as a factor of safety since there may be some variation in the cleaning process.

### REDDISH-BURLINGAME TEST FOR "COLD STERILIZATION"

The average number of aerobic spores found on surgical, dental, and veterinary instruments after use in practice was found to be 2, and the maximum number found on any instrument tested was 9. Based on these findings the following method is suggested for determining the practical sporicidal activity of chemical solutions: spread on Gillette-type razor blades approximately 1000 unheated spores of *B. anthracis* from a 7-day old culture grown on nutrient agar at 20° C of the following composition—0.5 per cent Difco beef extract, 1.0 per cent Armour peptone, 0.5 per cent NaCl, 1.5 per cent agar, adjusted to pH 7.4, in distilled water. Allow the inoculated blades to dry at 37° C for 1 hour in a sterile clay top petri dish and then expose to the action of the germicide being tested for 10, 20, and 30 minutes at 20° C. At the end of these periods wash the blades in sterile broth of the above composition for 1 minute and then transfer to 30 ml of fluid thioglycollate medium and incubate 3 days at 37° C. A satisfactory sterilizing solution should kill the spores of *B. anthracis* under these conditions within 20 minutes.

Practical tests with sterilizing solutions of known merit have shown this test to be satisfactory and that the results can be interpreted directly in terms of practical value. A margin of safety is

incorporated in the test since 100 times more spores are used than the maximum found on dirty used veterinary instruments. This is a simple practical performance test for chemical solutions recommended for the "cold sterilization" of previously cleaned surgical instruments.

Spaulding (1939), however, although admitting that surgical instruments are first cleaned before sterilizing, considers it safer to test chemical solutions for the purpose by adding blood or pus with spores to the instruments and testing them both wet and dry. This, evidently, would simulate the worst surgical conditions. Spaulding also found that the average number of spores found on used surgical instruments was 2, although the greatest number per instrument was 15.

### SPAULDING TEST FOR CHEMICAL STERILIZATION OF SURGICAL INSTRUMENTS

Eight test organisms are recommended, including 5 non-spore formers and 3 sporulating species, *B. anthracis*, *Clostridium tetani*, and *Clostridium welchii*. Detachable surgical knife blades are immersed in mixtures of individual test cultures and blood or pus and used in the test both wet and dried. The contaminated blades are then exposed to the germicidal solutions for definite time periods and then subcultured into broth. Control tests in sterile saline instead of germicide are included in each test and total counts and spore counts determined. The time periods of exposure specified are 1/2, 2, 3, 5, and 10 minutes for the non-sporulating test organisms and 5, 15, and 20 minutes and 1, 2, 4, 8, and 18 hours for the spore formers. Special media suitable to each test organism were employed for the test cultures and subcultures and adequate precautions were observed to prevent bacteriostasis in subcultures after exposure to germicides.

The method is useful in testing for sporicidal activity in the presence of organic matter, blood, and pus, but does not simulate customary surgical practice in which instruments are cleaned before sterilizing. It is not a practical performance test for chemical sterilizing solutions as they would be used in practice, but can be used to study the effect of organic matter on sporicidal agents.

A similar situation exists with respect to the disinfection of oral thermometers. Sommermeyer and Frobisher (1952), in a study of the effectiveness of certain germicidal solutions applied to mouth thermometers, emphasized the importance of wiping or cleaning such thermometers before immersing into disinfectant solutions.



When this is done exposure to such germicides for 10 minutes is usually sufficient for the purpose.

Another factor of considerable importance is the prevention of false readings due to bacteriostasis in the subcultures. Klarmann and Wright (1950) have demonstrated this quite clearly by testing the sporicidal activity of quaternary ammonium compounds in the presence and absence of blood serum. When blood serum was added to the subculture medium it neutralized the quaternary carried over and as a result allowed the unkilld spores to grow, whereas without this neutralizer growth did not occur, indicating apparent destruction of the spores. When tested without the neutralizer the spores were apparently killed within 10 minutes, but when the serum was used in the subculture medium the spores were not killed within 30 minutes and even, with a further modification, within 24 hours. This is contrary to the results obtained by Lewison (1950) when quaternary solutions were tested without the use of neutralizer in the subculture medium. The same difference in results was obtained when *M. pyogenes* var. *aureus* was used as the test organism. Without the use of serum neutralizer this organism was apparently killed within 15 minutes, whereas when serum was added to the subculture medium growth occurred after 120-minute contact.

Another *in vitro* sporicidal test was employed by Brewer (1939) in which large numbers of anaerobic spores were exposed to the action of the sterilizing agents in closed centrifuge tubes. The mixture was rotated in such a way that complete contact between germicide and test organism was assured. While this test was suitable for the special purposes involved in the study of organic mercurial compounds, it is not a practical performance test and was not recommended for this purpose. Although not a test for chemical sterilizers, Brewer (1950) made use of suitable *in vivo* tests to prove the effectiveness of organic mercurials as antiseptics under conditions of use in which spores of *Cl. tetani* were used. This is a practical performance test of a different character from those considered here, but was found most useful for demonstrating clinical effectiveness of a class of antiseptics.

## GENERAL CONSIDERATIONS

It is evident that *in vitro* methods for testing sporidical effectiveness of chemical solutions recommended for the "cold sterilization" of surgical instruments should be tested by a performance test procedure. It is also clear that such tests should be conducted on previously cleaned instruments or clean razor blades. Directions

for use of such sterilizing agents recommended for this purpose should clearly state that the instruments should be cleaned before treating with the preparation. The time period of treatment should also be indicated according to the results obtained from such practical performance tests.

Because of the variety of conditions under which surgical, dental, and veterinary instruments are used, it would be almost impossible to devise a single test method simulating all such conditions of use. If instruments are cleaned before sterilizing, as is customary, the Reddish-Burlingame method is suitable, but if the instruments are not cleaned beforehand clotted blood, dried serum, and pus should be present on the test material as in the Spaulding test method. Both methods are suitable for their respective and specified purposes. Since there is no standard method for testing for sporicidal activity of chemical sterilizers, one or the other of these methods may be used for the purpose according to the conditions under which they will be used.

## TESTS FOR STERILITY

The most acceptable and most widely used methods of testing for sterility are the official tests of the United States Pharmacopeia. By permission received from the Board of Trustees of the United States Pharmacopeial Convention these methods are presented here as they occur in the U. S. Pharmacopeia XIV (1950).

### Sterility Tests for Liquids and Solids

#### STERILITY TEST MEDIA

Test Media may be prepared as follows or dehydrated media of the same composition may be used for the preparation of official culture media provided they yield products conforming to those that are presented below.

#### I. FLUID THIOGLYCOLLATE MEDIUM

<i>l</i> -Cystine . . . . .	0.75 g
Agar, granulated (moisture content not in excess of 15 per cent) . . . . .	0.75 g
Sodium Chloride . . . . .	2.5 g
Dextrose . . . . .	5.5 g
Water-soluble Yeast Extract . . . . .	5 g
Pancreatic Digest of Casein . . . . .	15 g
Sodium Thioglycollate . . . . .	0.5 g
or Thioglycollic Acid . . . . .	0.3 ml
Resazurin Sodium Solution, 0.1 per cent, freshly prepared . . . . .	1 ml
Distilled Water . . . . .	1000 ml

Mix the *L*-cystine, agar, sodium chloride, dextrose, water-soluble yeast extract, and pancreatic digest of casein with 1000 ml of distilled water, and heat on a water bath until solution is effected. Dissolve the sodium thioglycollate or thioglycollic acid in the solution, and, if necessary, adjust the solution with 1 *N* sodium hydroxide so that, after sterilization, it will have a pH of  $7.1 \pm 0.1$ . If filtration is necessary, reheat the solution without boiling, and filter through moistened filter paper. Add the resazurin solution, mix thoroughly, place the medium in suitable culture tubes, and sterilize in an autoclave at  $121.5^\circ$  (exhaust line temperature) for 20 minutes. The autoclave temperature should be reached within 10 minutes. Cool at once to  $25^\circ$ , and store the medium, preferably between  $20^\circ$  and  $30^\circ$ , protected from light.

For 15 ml of medium use tubes preferably  $20 \times 150$  mm, and for 40 ml of medium use tubes preferably  $25 \times 200$  mm.

*Note*—Do not use this medium if evaporated to an extent affecting its fluidity or if more than the upper one-third has changed to a pink color. However, one reheating on a water bath, until the pink color disappears, is permitted.

## II. ALTERNATE FLUID MEDIUM

<i>L</i> -Cystine	0.05 g
Sodium Chloride	2.5 g
Dextrose	1.1 g
Water-soluble Yeast Extract	5 g
Pancreatic Digest of Casein	15 g
Sodium Thioglycollate	0.5 g
or Thioglycollic Acid	0.3 ml
Distilled Water	1000 ml

Heat the ingredients in a suitable container on a water bath until solution is effected. Mix thoroughly, and, if necessary, adjust the solution with 1 *N* sodium hydroxide so that, after sterilization, it will have a pH of  $7.1 \pm 0.1$ . Filter, if necessary, place in Smith fermentation tubes, and sterilize in an autoclave at  $121.5^\circ$  (exhaust line temperature) for 20 minutes. The autoclave temperature should be reached within 10 minutes.

The Smith fermentation tubes shall hold 30 ml each and have sufficient air space remaining in the bulb to hold all the liquid displaced from the long arm. The fermentation tubes are preferably closed with loosely fitting cylindrical glass caps.

*Note*—Certain products are turbid or otherwise unsuitable for culturing Fluid Thioglycollate Medium (Medium I) because of its viscosity. Alternate Fluid Medium (Medium II) is acceptable in



place of Fluid Thioglycollate Medium, provided it is used in Smith fermentation tubes, and, just prior to use, heated in a tilted position in a boiling water bath to drive the dissolved oxygen from the medium in the closed arm.

### III. SABOURAUD LIQUID MEDIUM (MODIFIED)

Peptone . . . . .	10 g
Dextrose . . . . .	20 g
Distilled Water, . . . . .	
To make . . . . .	1000 ml

Dissolve the peptone and the dextrose in the distilled water with gentle heat. Adjust the medium so that, after sterilization, it will have a pH of  $5.7 \pm 0.1$ . Filter, if necessary, place in culture tubes, and sterilize at  $121.5^{\circ}$  (exhaust line temperature) for 20 minutes. The autoclave temperature should be reached within 10 minutes in the closed arm.

### TECHNIQUE FOR CONDUCTING STERILITY TESTS

The test procedures presented in this chapter are suggestions for manufacturing control, and should be supplemented occasionally by more exhaustive tests, using more samples, more tubes, and different media and methods.

All sterility tests should be conducted by competent personnel. Rigid aseptic precautions should be observed throughout and all manipulations conducted preferably in a dustproof room supplied with filtered air under positive pressure. Operations should not be conducted under direct exposure to ultra-violet light or in areas under aerosol treatment.

*Controls.*—Prepare the following controls (*a* and *b*) simultaneously when testing any material for sterility:

(*a*) Test each lot of Medium I or of Medium II for its growth-promoting and oxidation-reduction qualities, using one or more bacteria that are exacting in their growth requirements. At the end of the incubation period used for the sterility test, less than 50 per cent of the medium in the tube shall have changed color.

(*b*) Confirm the sterility of each lot of sterilized test medium used in sterility tests.

(*c*) If the product under test may be bacteriostatic when cultured as directed, inoculate at least 5 per cent of all negative tubes of Medium I or II with 1 cc of a 1 to 100,000 dilution of an 18- to 24-hour fluid culture of *Clostridium novyi*, and incubate at  $32^{\circ}$  to  $35^{\circ}$  for 3 days. In a similar manner inoculate 5 per cent of all

negative tubes of Medium I or II with 1 ml of a 1 to 100,000 dilution of an 18- to 24-hour fluid culture of *Escherichia coli* and incubate at 32° to 35° for 3 days. Also inoculate 5 per cent of all negative tubes of Sabouraud Liquid Medium (Modified) with 1 ml of a 1 to 1000 dilution of a 72-hour Sabouraud Liquid Medium (Modified) culture of *Monilia albicans*, and incubate at from 22° to 25° for 3 days. Failure of growth is evidence that bacteriostatic or fungistatic agents carried over in the test material may be responsible for the negative results.

*Containers to be Tested.*—The number of final containers to be tested to insure the sterility of all units in a single sterilizing or filling operation is not indicated. However, a sufficient number from each finished lot should be taken in a representative manner and tested for sterility.

In addition to the sterility tests of the product in final containers, proper sterility control must be predicated upon the demonstration of the efficacy of each manufacturing process designed to attain the sterility of the product.

#### PROCEDURE FOR TESTING LIQUIDS

*Opening Containers.*—Remove dust particles from ampuls with sterile cotton saturated with alcohol or other suitable volatile agent. With the aid of sterile forceps, flame the ampul adequately, but avoid undue heating of the contents. Open the ampul using a sterile file.

In the case of containers closed with rubber caps or other closures, remove dust particles from the surface of the cap with sterile cotton saturated with alcohol, and treat adequately with iodine tincture or with other suitable substances. If the liquid in the container is under vacuum, admit sterile air by means of suitable sterile equipment such as a syringe needle attached to a syringe barrel filled with sterile non-absorbent cotton.

*Culturing.*—Remove the contents for culturing with a sterile pipette or with a sterile syringe and needle.

Plant one or preferably more tubes of Fluid Thioglycollate Medium and Sabouraud Liquid Medium (Modified) with portions of the liquid from the container being tested. Except in the case of liquids which are inherently bacteriostatic or contain bacteriostatic agents, the amount of inoculum and the volume of medium shall be varied according to the content of the container as follows:

Content of Container	Minimum Volume of Inoculum	Volume of Medium
Less than 2 ml	0.5 ml	7.5 ml
Less than 20 ml	1 ml	15 ml
From 20 ml to 50 ml	5 ml	40 ml
More than 50 ml	10 ml	40 ml

Liquids which are inherently bacteriostatic, or contain bacteriostatic agents, shall be treated with a suitable, sterile inactivating agent, or diluted beyond the bacteriostatic level by dividing the inoculum into a greater number of tubes so that growth will not be inhibited.

Mix the liquid thoroughly with the medium. If the test material is an oil, shake the mixture sufficiently at the time of planting and at frequent intervals during the incubation period to disperse the oil in the medium. Incubate the tubes at a temperature between 32° and 35°, and examine for growth at the end of the second, fourth, and seventh days after planting, being careful to look for possible aerobic growth at or near the surface of the medium.

When the liquid to be tested renders the medium turbid so that final interpretation of growth cannot be made at the end of 7 days, confirm the presence or absence of growth by microscopic examination of stained smears fixed with methanol, or make transfers from the tubes originally planted to tubes of fresh medium, and incubate these tubes at 32° to 35° for not less than 3 days.

Incubate cultures of material Sabouraud Liquid Medium (Modified) at 22° to 25° for at least 14 days for molds and yeasts. Confirm cultures showing macroscopic growth by a microscopic examination of stained smears.

#### PROCEDURE FOR TESTING SOLIDS

*Opening Packages and Containers.*—For purified cotton, gauze, surgical dressings, and related products, flame, with care, the immediate carton, package, container, or one of the margins, if an envelope. For sutures, make a file line in the center of the suture cube, or about 10 mm above the tubing fluid, place the tubes in a suitable, effective disinfectant solution for 24 hours, remove with sterile forceps, and place them between sterile towels. As an alternative method of sterilization, flame the tubes, preferably in a wing flame, but avoid heating the contents. Break the tube at the filed line, preferably by holding a red-hot, curved wire against it. For crystalline or powdered solids, proceed as directed for *Opening Containers* under *Procedure for Testing Liquids*.

*Culturing.*—From each carton, package, and similar container remove, with sterile forceps, and with sterile scissors, if needed, duplicate or triplicate portions of the material to be tested, from various locations within the roll of purified cotton, gauze, surgical dressing, or related material, preferably from the outer end, the center, and the core. Flame the forceps and the scissors thoroughly between successive transfers. Each portion of purified cotton shall weigh approximately 0.25 Gm, and each portion of gauze and



related material shall be approximately 3 sq in in area. For crystalline or powdered solids transfer with aseptic technique 10 per cent of the content of the container, but no less than 100 ml, but if the content is 100 ml or less, transfer all. Transfer these portions of the material, as rapidly as possible, to the necessary number of tubes, each containing 40 ml of Fluid Thioglycollate Medium, and also transfer portions to tubes each containing 40 ml of Sabouraud Liquid Medium (Modified). Transfer entire sutures to culture tubes each containing 40 ml of Fluid Thioglycollate Medium, and to culture tubes each containing 40 ml of Sabouraud Liquid Medium (Modified).

Adhesive absorbent gauze, sutures, or other solids which are inherently bacteriostatic, or contain bacteriostatic agents, shall be treated with a suitable, sterile, inactivating agent, or brought beyond the bacteriostatic level by planting in a greater volume of culture medium, so that growth will not be inhibited.

Incubate cultures of sutures at 32° to 35° for 15 days before negative results are recorded. Incubate other solids at 32° to 35° for 7 days before negative results are recorded.

Incubate cultures of materials in Sabouraud Liquid Medium (Modified) at 22° to 25° for at least 14 days for molds and yeasts.

Confirm cultures showing macroscopic growth by a microscopic examination of stained smears. Fix smears from Fluid Thioglycollate Medium with methanol.

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## 8

# BACTERIAL RESISTANCE AND DYNAMICS OF ANTIBACTERIAL ACTIVITY

## INTRODUCTION

RESISTANCE to noxious influences has interested biologists from the beginning of the Christian era to the present time. The inability of Mithradates to commit suicide by poison because of resistance acquired by adaptation fired the imagination of the romanticists; the development of resistance in cancers to radiations and chemotherapy will have serious consequences for a considerable number of our practical-minded contemporaries. The habituation to alcohol and narcotics in man is well known and has its parallel in insects resistant to DDT. Microbiologists became aware of the variable resistance of microorganisms during the 1880's and the facility with which organisms could be trained to resist the chemical inhibitors, of interest in the early development of bacteriology, is reviewed by Regenstein (1912). Such early papers merely record the observation that resistance could be acquired if increasing amounts of phenols, heavy metals, salts or dyes were added to serial cultures.

The development of chemotherapy by Ehrlich led to the observation that trypanosomes could develop resistance *in vivo* to dyes and arsenicals. Extensive investigation of this problem in trypanosomes preceded the advent of clinical bacterial chemotherapy by almost 30 years. With the advent of sulfonamides bacterial drug-resistance became a major line of research. Garrod (1950) writes of this period: "Gonococci encountered in clinical practice were developing resistance at such a rate that it was fortunate that penicillin appeared to replace sulfonamides." Whether the future

of bacterial chemotherapy requires the discovery and introduction of new drugs as old ones become useless is not evident from existing data but such a possibility is recognized.

Meanwhile those concerned with antibacterial actions other than those of chemotherapeutic agents have also expanded the original observations on resistance. When employing the standard tests for antibacterial activity a lack of reproducibility was observed which could be ascribed to variation in the inoculum of microorganisms. Certain species were recommended as the best test organisms, and in critical tests even special strains of these species were required; these must be cultured under specified conditions so that the resistance will fall within definite limits. Unfortunately resistance to one agent has no predictable quantitative relationship to resistance to other unrelated agents so that standardization of this factor has not been solved except where one can use a closely related antibacterial substance as a reference.

The attempt to elucidate the mode of action of antibacterial agents is closely tied in with resistance phenomena. Most noxious influences applied vigorously to the delicately organized and highly reactive protoplasm of the living cell will introduce numerous modifications deleterious to the organism. An antibacterial action will succeed when, of all the functions which could be modified by the antibacterial agent, the most sensitive one necessary to growth or survival of the cell is unable to proceed. Methods for testing activity and resistance accentuate this point since the end-point employed in such experiments is that least amount of antibacterial action, in intensity or time of application, which will just bring about the inhibition or death. A study of resistance is thus by methodology a study of mode of action against the most sensitive site. It seems reasonable that organisms placed in 5 per cent phenol will be killed or inhibited because dozens of cellular activities are destroyed; yet in "mode of action" studies emphasis is on the limiting case—on that organism which just fails to survive or grow because a single necessary function failed to escape destruction. Modification of this single function will make an organism more resistant than its fellows, and in a study of resistant organisms this change and therefore the site of the antibacterial action may be revealed.

Knowledge of the variations in natural resistance of the various species has been employed to good advantage in the development of differential culture media and in the selection of the proper disinfectant for the particular purpose where the nature of the offending contaminant is known.



## DEFINITIONS AND CONCEPTS

*Sensitivity* and its antonym, *resistance* refer to the quantity of an antibacterial principle that is tolerated. These terms are applied in reference to both bacteriostatic and bactericidal action. They are quantitatively evaluated in terms of intensity (concentration) or time of application.

The *wild type* or sensitive strain is a strain in which the investigator has made no attempt to induce resistance to the antibacterial agent. A sensitive strain of *Escherichia coli* may tolerate more penicillin than most resistant strains of the genus *Bacillus*. This is termed *natural resistance*.

*Acquired resistance* is that which results in response to manipulation of the wild type. *Induced resistance* usually implies the application of a *mutagenic agent* to the culture before the selection or training process.

*Complete resistance* of an organism is the maximum that can be obtained with repeated trials of procedures leading to acquired resistance. Such organisms are said to be *fully resistant*.

*Partial resistance* describes a condition between that of sensitive and complete resistance. In developing complete resistance is usually necessary to pass through stages of partial resistance.

*Cross resistance* is exhibited when an organism that has been trained against one drug becomes resistant at the same time to another drug. When there is a lack of cross resistance the term *specific resistance* is used.

*Dependence* is encountered when resistant organisms develop a condition in which they are unable to grow in the absence of an inhibitor.

*Training* involves repeatedly subjecting the organism to the maximally tolerated antimicrobial agency to secure resistance. In most cases it appears to be a clumsy method of selecting resistant mutants and eliminating non-resistant organisms rather than a procedure for modifying all the cells in the population.

*Adaptation* is a general term for the numerous processes by which the organisms become better fitted to the environment.

*Reversion* to wild type refers to loss of resistance when resistant organisms are cultured in a normal medium. This is usually regarded as due to the selective advantage which wild type organisms (arising by back-mutation) have over resistant mutants when in their natural environment. In some cases reversion occurs in a single subculture while in others resistance persists for many transfers in the absence of the inhibitor.

*Resistance to Bactericidal Action* is exhibited when the graph of the log of the survivors plotted against time gives a line with less slope than that of the wild type.

*Collateral Sensitivity* is decreased resistance to one agent which accompanies increased resistance to another.

## METHODS

*Resistance to Bacteriostatic Action.*—Bryson and Szybalski (1952) have reviewed the procedures for obtaining resistant mutants to bacteriostatic agents. These usually involve serial subcultures with increasing concentration of antibacterial agent in liquid or solid media; in this way a stepwise increase in resistance can be obtained. The steps can be bigger if the inoculum is large since in any inoculum the individuals widely different from the normal are rare. With some inhibitors, such as streptomycin, large increases can be obtained in a single step while with others such as salt or phenol only very small increases can be obtained. If a single mutation changes a wild type to a fully resistant organism even though such a mutant occurs only once in 10 million organisms there will be 1 in every drop of inoculum planted and only 1 transfer will be needed to develop complete resistance. On the other hand, if full resistance results only from several separate mutations, even if each of these occurs once in every 10 million organisms, the chance of having even two of them occurring in a single organism is one ten millionth times one ten millionth which is so rare that that many organisms are unlikely to be studied in any set of experiments. Therefore, one must select for the first mutation and, after raising a culture of it, select for the second. The probability of finding rare mutants can be enhanced by subjecting the organisms to mutagenic chemicals or radiations to increase the mutation rate.

Bryson and Szybalski have devised continuous procedures to permit the selection of resistant organisms with less utilization of labor or material. The *gradient plate method* involves a petri plate in which 20 ml of nutrient agar is allowed to harden while the plate is in an inclined position. The plate is then returned to horizontal and a layer of agar containing the toxic agent is poured on top of the hardened wedge of nutrient agar. Downward diffusion of the toxic agent then gives a continuous gradient of toxic concentrations across the plate, and only the resistant organisms from a culture streaked on the surface will grow at any distance from the edge where there is a thick layer of agar lacking antibacterial action. As soon as such colonies appear they may be spread in the direction of greater concentration of inhibitor so that in a few

days with several streakings very resistant organisms can be obtained from a single plate. In principle this is the same as the cup plate, the assay cylinder, or the filter paper disk, but it gives a wider space to work and a much more gradual increase in concentration of the toxic substance along the gradient axis of the plate. A turbidostatic selector was also developed for the automatic selection of mutants in broth cultures by a continuous culture device which feeds increasing concentrations of toxic substance and fresh broth as the culture develops. A simple and ingenious modification of this principle is reported by Matney and Mefferd (1952) who used an antibiotic which increased in activity with increased acidity, together with a medium in which the test organism produced acid from sugar. The amount of antibiotic added initially was set at that level so that only a few partially-resistant mutants could grow; as they grew they produced slight acidity which made the antibiotic more active so that only still more resistant mutants arising from the partial mutants would grow. The final culture was much more resistant than would have resulted if the pH were held constant.

*Resistance to Bactericidal Action.*—In general methods involving the destruction of microorganisms, where logarithmic death prevails, do not select out resistant members of the population as the last survivors. Although the resistant organisms die at a decreased rate, because of their fewer numbers they are eliminated early in the killing process (Wyss, 1951). However, as implied by Lahn (1945) and demonstrated by Chaplin (1951) when the death curve varies from the usually logarithmic pattern there is strong evidence to suspect that a resistant strain may be isolated by repeatedly culturing the last survivors. Even where killing follows a definitely logarithmic pattern it is sometimes possible to isolate strains resistant to killing. Wyss (1950) has observed that with some organisms and antimicrobial actions, resistance to bacteriostatic action is accompanied by resistance to bactericidal action. Following this lead, Berger and Wyss (1953) isolated strains resistant to the killing action of phenol, but in order to demonstrate this resistance to killing it was necessary to grow the cell crop in a medium containing a sub-inhibitory concentration of phenol.

Witkin (1946) has developed a procedure with which it is possible to isolate organisms resistant to killing by radiation even if this killing is logarithmic; with modifications this may be applied to killing by other agents. The procedure involves seeding a plate with organisms and permitting microcolonies of about a thousand cells each to develop before subjecting the plate to irradiation. Each microcolony will be killed in a more or less logarithmic fashion



with slopes determined by the resistance and if any consist of all resistant organisms there will be a few survivors when all organisms in non-resistant colonies are dead. On further incubation visible colonies will grow from the resistant survivors. This simple procedure permits the ready examination and testing of thousands of individual populations (the colonies) to observe inherited differences in killing rates.

## NATURAL RESISTANCE

Natural resistance of microorganisms to deleterious agents appears to be inherent in the genetic makeup of the organisms and may have arisen in a variety of ways. Certain organisms acquire resistance incidental to their acquiring other characteristics useful to their survival. For example, Bisset (1950) suggested that endospores have become resistant to heat and chemicals far beyond a range which could have been tested in evolutionary development because this resistance is only incidental to some characteristic more useful in evolution. On the other hand the selective action of the bile has eliminated from the gut of mammals all organisms except those tolerant to some degree of surface active chemicals. Organisms which can synthesize the vitamins and amino acids necessary for growth are less at the mercy of substances that interfere with diffusion of organic materials from the medium into the cell. Finally, our method of staining puts into one group those organisms that fail to firmly react avidly with basic dyes and therefore are more resistant to them and to other substances of similar reactivity. Even in the fragment of the world of biological activity discussed here, the futility of postulating or searching for any single mechanism common to the various phenomena of organic adaptation is obvious.

Microorganisms are complex systems adapted to continued survival in their available environment. When stresses are brought to bear upon the system three possibilities are revealed: (1) the system breaks down and the organism dies or fails to leave progeny for the continuation of its line; (2) the system develops means of resisting; and (3) the system changes by accommodating itself temporarily or permanently to the presence of the undesirable influence. This chapter considers the resistance and accommodation which microbes exhibit to destructive influences which we impose on them in our efforts to destroy them or control their activity.

Each living microorganism now in existence owes its presence on the contemporary scene to the fact that it has met and passed innumerable tests for survival. Its mechanism of heredity has

passed from one generation to another those qualities which it now exhibits. We say these organisms are adjusted to present environment because they have met similar environments in the past and those that failed to fit were eliminated. However, no organism can be exactly and rigidly adjusted to its environment since no two organisms will ever be confronted with exactly the same set of conditions in space and time. Consequently, there is a limit beyond which heredity can not prefit the organism since it must be adjusted to a normal range of conditions. The major lines are drawn but the details are left to be filled in; the organism can make adjustments within the fixed limits set by its heredity. Sometimes these limits are wide such as the thermal tolerance of facultative thermophiles; in other instances it is restricted such as the pH tolerance of the treponema of syphilis. Weiss (1949) suggests that there is only one generalization that can safely be drawn and that is that the latitude left to "on the spot" adaptation is extremely narrow as compared with the wealth of inherited adaptedness of evolutionary origin. The ratio between the two varies from species to species and from function to function.

Some of the seeming adaptations to meet minor environmental changes on the part of the organism are based on the possession of several procedures to accomplish the same objective. For example organisms have several sets of enzymes so that sugar breakdown may proceed by more than one pathway. Again this is by evolutionary predesign for if interference with a particular process occurred sufficiently often in nature the organism having a spare process would have a distinct advantage in survival. Organisms with both pathways may develop the uninterrupted one to a greater degree; in general, we may expect adaptation to be quantitative in nature in that much of it will be "more or less" rather than qualitative in character.

Living cells contain by virtue of their hereditary make-up certain enzymes which are prefitted to act on matched substrates. However, if we permit microorganisms to contact certain unfamiliar substances new enzymes may be formed. There are indications that they are formed at least in part from existing enzyme proteins in response to the presence of a new substrate. The substrate has a molding effect on the configuration of the enzyme that is analagous to the formation of antibodies from blood proteins in response to the presence of antigen. These are called *adaptive enzymes* and play an important role in the life of microorganisms especially as the amounts formed will cause decrease in other enzyme functions by competing for protein from the pool available for enzyme formation. One can picture how the presence of adaptive enzymes would

modify the appearance of a drug-resistant microorganism in a variety of ways. Suppose the destructive influence inhibits the reaction which converts A to B. The presence of excess A may induce the formation of more of the inhibited enzyme which means that the cell will now withstand more of the destructive influence. Or excess A may induce the formation of a different adaptive enzyme so B may be formed by another pathway. A thorough study of such resistant organisms may show them to be different from the wild type in many respects since the new enzyme formed is at the expense of some other enzymes in the cell; the consequences of the one change may be exhibited many fold and often far removed from the original site of the change.

Variations in the natural resistance as encountered by the microbiologist may be due to changes in the organism but in every day practice one often encounters variations which are due to changes in the culture medium. These changes may be brought about by the microorganism or they may be introduced by design or inadvertently by the investigator. It is widely recognized that standardization of medium is necessary to obtain uniform results and this is discussed in preceding chapters. The factors to be considered are the rate of growth, and total crop, the pH, the state of oxidation, and the presence of antidotes. In testing the lethal action of an antibacterial substance any modification of the medium that gives a higher yield of cells or a faster rate of growth will generally exhibit a greater resistance by the methods used in testing. It takes more phenol to kill 1 billion cells per ml than 1 million cells; the amount of streptomycin that just inhibits organisms in a medium where they divide once per hour will have to be increased if a little yeast extract is added so as to make them divide every half hour. Non-specific interference of amino acids is observed for almost any inhibitor when tested in a minimal culture medium. The pH of the medium is set by the investigator and it is modified by the organisms during growth. Some substances such as streptomycin are more active at high pH values and are most often tested at pH 8.0; in the presence of a sugar from which the organisms form acid the activity is decreased. On the other hand, high peptone content may increase activity by the raising of the pH due to splitting out of ammonia. Other substances such as propionate are more effective at low pH values and an organism will appear more sensitive on a high sugar, low peptone medium. Burnett *et al.* (1952) have shown that the radiation resistance of microorganisms is increased by any factors in their growth medium which lower the oxidation state of the culture; the presence of readily oxidizable substrate, reducing agents such as thioglycollic acid or the mechanical removal of oxygen lowers the rate of killing markedly.



Klarmann (1950) has reviewed the role of antagonisms showing that the action of a variety of antibacterial substances is reversible and discussing the relationship of this phenomenon to the problem of performance of individual agents. This is relevant to the discussion here since these factors modify the apparent resistance of the microorganisms. In summary of this section, in reports in the literature purporting to measure the resistance to the germicidal action of some chemical, the actual measurement often concerns itself mainly with a disinfectant-wasting side-reaction unrelated to the interaction of chemical and organism.

Curran (1952) reviews the literature on variation in resistance of bacterial spores citing numerous references on factors which lead to increased resistance. These fall into two categories, those intrinsic or inherent in the cell and the extrinsic factors which operate from the outside. The extrinsic factors which may enhance or diminish resistance operate in (1) the environment in which the spores are formed, (2) the storage conditions and (3) the resistance test including the subculture medium. High temperatures during sporulation, the presence of fatty acids in the culture medium, or the presence of optimal amounts of metallic ions result in spore crops that are significantly more resistant to thermal destruction and in some cases to chemical disinfection. While such factors are readily controlled under laboratory conditions, in applied bacteriology one deals with organisms whose resistance may be markedly influenced since in their natural habitat a much greater number of environmental agencies are acting on the spores and in an ever changing succession.

Generally speaking, organisms that survive drastic killing influences are more fastidious in their nutritive requirements; the apparent resistance of a culture is increased if rich broths carefully adjusted to ideal pH, O-R and temperature conditions are used for subculture. They also become more sensitive to toxic substances in the subculture media and the incorporation of adsorbents such as starch or charcoal will reveal survivors which would have appeared non-viable in ordinary broth. This is observed with organisms injured by radiations as well as by heat and chemicals. An extreme example of effects of this nature is that greater resistance to killing by ultraviolet light is observed when the survivors are plated in a well-lighted laboratory rather than in semi-darkness. This is due to photoreactivation by visible light of organisms which had been "killed" by the ultraviolet (Kelner, 1949). Latarjet and Caldas (1952) observe a similar restoration upon the addition of catalase and certain other chemicals.

## INDUCED RESISTANCE

*Origin of the Resistant Mutants.*—Microorganisms present a special problem for the investigator in that we are able to study the behavior of an individual organism only by studying the behavior of its progeny. It is often difficult to determine if a change in a population was by adaptation in existing organisms or by selection of the descendants of a mutant organism in the original population. With a number of antiseptics there is strong evidence supporting the thesis that the resistance arises as a mutation in one out of the millions of cells comprising the initial sensitive population before it ever had contact with the antiseptic. The addition of the inhibitor then merely inhibits the rest of the organisms and permits the progeny of the one resistant individual to grow resulting in a resistant population. Bryson and Demerec (1950) observed two procedures for the development of resistance to antibiotics. With streptomycin, high resistance can be brought about gradually by a series of multiple mutations or at once by a single genetic change. Because of this latter possibility streptomycin resistance is a serious problem to the clinician. Resistance to penicillin requires a series of multiple genetic steps; first-step mutants obtained from sensitive cultures by the selective action of the antibiotic provide material for the additional selection of still more resistant second-step mutants and thus complete resistance results from a series of selective steps. Terramycin, aureomycin, chloromycetin and neomycin appear to follow the same pattern as penicillin.

Several lines of evidence are available to support the mutational origin of resistance. The fluctuation test of Luria and Delbruck (1943) which has been developed by Lea and Coulson (1949) has been applied to resistance to numerous chemotherapeutic agents and in each case demonstrates the statistical occurrence of such mutants and supports their mutational origin. Lederberg and Lederberg (1952) have devised an ingenious technique which makes use of a velvet stamp pad for transferring a series of replicates of a petri dish seeded with organisms. When such replicate plates are made by proper techniques, it can be predicted which colonies on the plates to which no chemical is added will be the resistant ones. Thus, clones of resistant organisms arise and multiply and can be isolated in pure culture without exposing the population to contact with the inhibitor. Finally, organisms which have other characters associated with resistance can be isolated without previously contacting the inhibitor such as the biotin-independent *Micrococcus pyogenes* var. *aureus* (English and McCoy, 1951) that turned out to be resistant to streptomycin, highly pigmented staphy-

lococci (Catlin and Salle, 1951) which were often phenol resistant, and high acid producing *E. coli* that were resistant to streptomycin (Wyss, unpublished observations).

In spite of this evidence the possibility that resistance may develop also as a result of contact with the chemical is not without support. Interference with normal development may result in more errors in the exact duplication of genetic material so the mutation rate of partially inhibited populations may be higher than that occurring spontaneously. This would result in a greater likelihood that very resistant mutants would appear so that in some instances the true picture of the development of resistance may be a modification of mutation and selection which is accentuated by mutagenesis of the antimicrobial agents. Demerec, Bertani and Flint (1951) have shown that mutagenicity is not a specific property of any one group of chemicals but appears among widely different groups. Such substances may increase mutation rate (1) by acting directly on the gene itself or (2) by interfering with some metabolic pathway leading to gene synthesis thus causing inexact duplication of the gene in the daughter cell.

The long lag period in a medium containing drugs suggests the occurrence of enzymic adaptation as well as selection. This phase is reviewed by Barer (1951). Whether the enzymic adaptation accounts for the resistance, whether it makes the cells more liable to mutation and selection, or whether it must occur in the mutants after the mutation accident but before the mutants can grow to mature cultures, is not clear. Sevag and Rosanoff (1952) review the position of those who believe that drug-resistant members of a population may be derived from sensitive cells not by spontaneous mutation but in response to the direct chemical action of drugs. They suggest that the drug produces in living cells "certain specific configurational changes which would be compatible or incompatible with the specific configuration of the drug." This would account for the absence of general cross resistance between drugs. Sevag and Rosanoff suggest that the inhibitory action of a drug on a bacterium is the result of a chemical reaction between the two reactants. As a result the activity of the site for a similar reaction with the reagent may be abolished.

The interaction of mutation rate, selection and population pressure on the phenol resistance of cultures used in phenol resistance tests are discussed by Catlin and Salle (1951). Mutants resistant to the bacteriostatic action of phenol were isolated by Berger and Wyss (1953) which were also resistant to killing by phenol but only when grown in the presence of subinhibitory concentrations of the chemical. This might be described as adaptation superimposed on a mutation.



*Changes in Resistant Organisms.*—Wyss (1950, 1951) has analyzed the possible changes in resistant organisms that might account for the resistance:

1. Cytological changes.
2. Decreased penetration of the drug.
3. Increased destruction of the drug (or conversion to an inactive compound).
4. Increased concentration of a metabolite antagonizing the drug.
5. Increased concentration of enzyme utilizing this metabolite.
6. Decreased quantitative requirement for a product of the metabolite.
7. Alternative metabolic pathway by-passing the metabolite.
8. Enzyme changed in some quality such as relative affinity for drug.

In cytological changes encountered in resistant forms it is often difficult to assign a definite causal relationship to the aberrant form. Careful examination of organisms resistant to radiations suggests that these cells contain more nuclear material than the sensitive cells. In some cases they appear to contain more distinct nuclei and often they appear to be definitely multicellular. Where death of organisms requires destruction of all active nuclei (*i.e.*, if a single living nucleus can support the living cell which ordinarily has several) then, if nuclei die according to a logarithmic or progressive order, it is obvious that cells containing several nuclei will be harder to kill than those containing single nuclei. Bisset (1950) has shown that most microorganisms are multicellular by demonstrating the presence of cross walls hard to see by traditional staining procedures. Thus resistance to heat, ultraviolet light and chemicals occurs where increased multicellular or multinucleate cells appear as inherited traits in a population and can be selected by appropriate methods.

A convenient catch-all for otherwise unexplained effects in biology has been to dismiss them as modifications in the ability of a substance to penetrate into the cell. There are a number of resistance phenomena where the membrane clearly is implicated. Chaplin (1952) has isolated strains of *Serratia marcescens*, an organism ordinarily sensitive to 100 ppm alkyldimethylbenzyl ammonium chloride, which grew in 100,000 ppm of the chemical. Staining the resistant strain with fat stain showed a surface layer of lipid material which was absent in the sensitive strains. Extraction with fat solvents showed 31 per cent in the resistant organism and only 5 per cent in the sensitive organism. Treatment of the resistant cells with lipase removed the fat and rendered them much

more sensitive. In an earlier paper, Chaplin, (1951) showed that the original population was non-homogenous as demonstrated by the sigmoid death curve, and that cultivation in the absence of the inhibitor resulted in immediate reversion to non-resistance. Resistance is presumed to be due to the elaboration of a lipid which is retained on the cell surface and gives resistance (a) by excluding the antibacterial agent from the surface (b) by reacting with it, or (c) by rendering the surface capable of withstanding the disruptive surface force of the disinfectant. This appears to be an example of mutation to resistance which can occur only in certain gram negative organisms and which is very disadvantageous to the organism except in the presence of the inhibitor. Chaplin suggests that the substance is a lipid-protein complex that occurs in gram negative but not in gram positive bacteria. Dubos (1944) ascribed the natural resistance of gram negative bacteria to a phospholipid complex which could be extracted from their surface but as yet no one has demonstrated an increase in this substance in resistant strains. The presence of an unusual phosphoric ester in gram positive bacteria has been described by Mitchell and Moyle (1950) and it is suggested that this substance may be associated with the penicillin sensitivity of those forms. Twenty per cent of the total phosphorous of the cells was found in this particular ester.

The relationship between the membrane and drug resistance may be a subtle one. Gale and Paine (1951) report that penicillin inhibits the formation of enzymes which transport glutamic acid across the membrane. Resistance may involve synthesis of such nutrients inside the cell so that transporting enzymes will be of diminished importance. Lack of penetration has been more satisfactory for explaining resistance in trypanosomes than in bacteria; the problem is reviewed by Work and Work (1948).

The destruction of the drug or its conversion to an inactive form is reported by Stearn and Stearn (1932) with crystal violet and acid fast organisms. The anomaly of the dye resistance of the gram positive mycobacteria is explained by clear zones surrounding colonies on crystal violet plates demonstrating the destruction of the dye. *In vitro* staphylococci regularly throw off mutants that produce penicillinase, an enzyme which destroys penicillin, but isolation of penicillinase producers from clinical cases is rare. Obviously other mechanisms of penicillin resistance exist and Gilson and Parker (1948) think that the penicillinase type of resistance is an artifact introduced by the method of testing for drug resistance. Penicillinase is an adaptive enzyme and arises in organisms capable of its production as a result of contact with penicillin. Thus, it is possible that its presence would never be observed except in

those organisms which are resistant by some other mechanism so that they may survive the contact with penicillin and produce penicillinase. This is consistent with the observation of Hahn and Wisseman (1951) that antibiotics suppress adaptive enzyme formation. It has been suggested that low levels of heavy metals too small to completely inhibit the metabolism of microbes will, in some cases, eventually permit growth since the cell is capable of producing reducing substances that will remove and detoxify the mercurials.

Catalase, alcohol dehydrogease, and formic hydrogenlyase are enzymes which destroy potential inhibitors but again, because of their adaptive nature, their presence in high amounts in resistant cells may be an artifact. It has been observed that a good part of the damage that results from irradiation is due to the formation inside the cell of oxidized substances such as peroxides. Clark (1952) has measured catalase activity of radiation resistant strains of *E. coli* and finds increased activity of the enzyme.

Increased concentration of the metabolite antagonizing the action of the drug accounts for some but not all sulfonamide resistance. Leskowitz, Fox and Raymond (1952) have isolated the anti-sulfonamide substance produced by resistant staphylococci and characterized it a *p*-aminobenzoic acid. It is believed that such a detoxification mechanism is wide-spread in nature but in many instances the problems of qualitative and quantitative identification of the small amounts of metabolite involved have thus far been insurmountable. The fact that some sulfonamide-resistant organisms exist which do not produce excess *p*-aminobenzoic acid proves that resistance may be by some other mechanism. Under some conditions pantothenic acid interfered with streptomycin inhibition and Wyss and Schaiberger (1953) have described a resistant strain of *E. coli* that contains greater amounts of the pantothenic-synthesizing enzyme.

Closely related to the production of increased competitive metabolite is the problem of the nutritional requirements of the organisms. In general, gram positive organisms are exacting in their requirements for external sources of amino acids and vitamins while gram negative organisms often synthesize most of these substances. This may be related to the generally greater resistance of gram negative organisms to a wide variety of antibacterial substances. McIlwain (1943) has found that the development of resistance to pantothenic acid analogues was associated with a decrease in nutritional exactitude, and Gale and Rodwell (1948) observed that a penicillin resistant mutant had acquired the ability to grow without an exogenous supply of amino acids. By a process of "training" staphylococci can be developed which grow in the absence



of most amino acids; such strains are more sensitive to inhibition by toxic amino acid analogues.

An understanding of sulfonamide bacteriostasis is helpful in explaining increased resistance by virtue of increased synthesis of amino acids, vitamins, purines and pyrimidines. An organism will fail to be inhibited by sulfonamide if PAB is added to the medium or if the products of PAB action are added. The products are folic acid, methionine serine, purines and pyrimidines. If some or all of these are supplied in adequate amounts the PAB becomes more effective in interfering with sulfonamide bacteriostasis. As a consequence of increased synthesis of any product of action of metabolite, a good supply of these substances at or near the site of their utilization will render a cell more able to resist the inhibitor of the metabolite.

The alternative metabolic pathway which may be used or even developed as a means of by-passing an inhibition has already been mentioned. Smith, Oginsky and Umbreit (1949) have concluded that streptomycin-resistant strains utilize a different pathway for disposing of the pyruvate formed in glycolysis. Sensitive strains condense pyruvate with oxalacetate, a process inhibited by streptomycin. The alternative pathway utilized by the resistant strains was not defined but several possibilities are evident. Kohn and Harris (1942) developed two distinct strains of sulfonamide-resistant *E. coli*. During a training period in the presence of methionine a strain developed a requirement for methionine; when resistance was developed in the absence of methionine the organism did not develop this requirement. Since sulfonamides normally inhibit methionine synthesis the latter organism must synthesize methionine by some new sulfonamide-resistant route.

An unusual opportunity to distinguish between these changes in resistant organisms arose when Davis and Maas (1952) observed that a single compound *p*-nitrobenzoic acid *PNB* was inhibitory to *E. coli* because it interfered competitively with two distinct enzyme systems, one involving *p*-aminobenzoic acid, *PAB*, (the same as involved in sulfonamide bacteriostasis) and *p*-hydroxybenzoic acid, *POB*, (a new bacterial growth factor). Other inhibitors are available that attack only one site and resistance at each site developed independently; yet an organism resistant at the *PAB* site (demonstrated by resistance to sulfonamides) still was inhibited by *PNB* because it still interfered with the *POB* site. This observation excludes the possibility of the lack of penetration of the drug or the destruction of the drug as an explanation of resistance because if the active drug can get at one enzyme site it ought to be able to get at the other. Resistance to various analogues of

PAB was observed to be partly or completely specific thus eliminating increase in enzyme or increase in metabolite as the mechanism of resistance. Resistant strains of organisms which require PAB for growth still require as much PAB as the non-resistant organism thus excluding increased efficiency of the PAB utilization or the opening of some by-pass mechanism as the explanation of the resistance. All data support the hypothesis of the presence of an altered enzyme protein in the resistant strain. The appearance in the microbial genetics literature of other examples of mutations in microorganisms which result in altered enzymes supports this view.

Another enzyme modification is reported by Yall and Green (1952) who found that ureases and dehydrogenases of *Micrococcus pyogenes* var. *aureus* are produced that are resistant to sulfhydryl (SH) inhibitors such as furacin, mercurials, and the trivalent arsenicals. These enzymes appear to have fewer or no reactive (SH) groups as contrasted with similar enzymes for the sensitive organism and they suggest that the presence of the SH group may not affect the activity of the enzyme yet it will make the enzyme liable to inactivation by mercurials, etc. In the same manner enzymes in heat resistant organisms are reported which are not denatured by temperatures that destroy the same enzymes in sensitive organisms (Millitzer, Tuttle and Georgi, 1951; Maas and Davis, 1952).

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## 9

# IODINE

## INTRODUCTION

IN 1776, L. G. Cadet, a French chemist, observed that a blue-green substance resulted from the treatment of kelp (seaweed ashes) with acids. However, it was not until the latter part of May 1811 (Gay-Lussac, 1814*a*) when another French chemist, Bernard Courtois, while washing kelp with sulfuric acid to destroy certain impurities, noticed violet fumes which condensed, caked up and corroded his copper equipment (Griffith, 1930). Courtois (1813) confided in two friends, C. B. DeSormes and N. Clement, who presented his report to the Institut Imperial de France on November 29, 1813.

Following Clement's reading of Courtois' paper, Gay-Lussac, Ampere, and Chaptal further studied this new substance. Davy, while in Paris, upon being told of Courtois' discovery, at once suspected it to be elemental in nature and soon confirmed his suspicions. Simultaneously, Gay-Lussac (1813) arrived at the same conclusions, presented his findings (Toraude, 1921), and the result was a tautological battle between Davy (1813*a, b*, 1814, 1816) and Gay-Lussac (1814*b*) as to the priority of their discoveries.

Gay-Lussac named the element "iode" in French after the Greek word meaning "violet-colored," due to the color of the vapors of iodine. Shortly thereafter Davy invented the English term "iodine" from the same root.

So far as is known, the first use of iodine in medical practice was its use as a remedy for goiter by Dr. William Prout in 1816 (Prout, 1834). Sir Andrew Halliday, on his return from a visit to Dr. Coindet in Geneva in 1819, recommended the use of iodine for thyroid conditions (Coindet, 1820), and in 1821 published a paper detailing the uses and advantages of iodine and its preparations (Halliday, 1821). Between 1820 and 1840 there followed an inter-

esting series of monographs on the external and internal uses of iodine for an astonishing variety of diseases.

The first specific reference to the use of tincture of iodine in wounds was made in 1839 (Davies, 1839; Boinet, 1865). Iodine was officially recognized by the Pharmacopeia of the United States in 1860 and iodine (as linementum iodi) first appeared in the British Pharmacopoeia in 1885 (Lancet, 1948).

Liebig reported on the antiseptic properties of iodine and Koch (1881) reported that iodine was capable of destroying the viability of anthrax spores within 24 hours.

Davaine (1873), in comparing the antiseptic action of iodine with other antiseptics of that time, found that a 1:20,000 solution of iodine attenuated the virulence of anthrax bacilli. Davaine (1880) also reported that a 1:170,000 solution of iodine placed in contact with anthrax-infected blood for a period of 50 to 60 minutes rendered the blood harmless upon injection into guinea pigs.

Sereins (1887) successfully treated two cases of human anthrax by the injection of iodine solution (1:100) around and into the anthrax pustule. This was confirmed by Brochin (1880). The earliest reference to the antiseptic use of iodine in surgery was published by Bryant (1884). He recommended the irrigation of wounds with a solution made by adding "10 drops of the liquor iodi per ounce of water." The earliest reports in the United States in which iodine was applied to flesh wounds were made in 1889 by Dr. Powell at Rush Medical College in Chicago (Niece, 1913).

According to a report by Allain (1895), in which the action of iodine on various microbes was discussed in detail, it was found that pathogenic bacteria were extremely susceptible to iodine. The cholera bacillus was the most sensitive and was killed by an iodine concentration of 1:200,000.

Gaucher (1903) reported the successful treatment of a human case of actinomycosis by the daily injection of 2 ml of 10 per cent iodine solution. Chassevant (1907) recommended iodine for the disinfection of the skin. The special usefulness of iodine as a skin disinfectant was reported by Grossich (1908). Other references are given later.

Since the latter part of the nineteenth century much has been written extolling the virtues of iodine as an antiseptic. This has been largely due to the fact that it fulfills a function that many other bactericides do not and cannot do. Iodine has been used in various forms as an antiseptic for the skin, wounds, and mucous surfaces of the body; for the sterilization of the air and of inanimate objects such as catgut and surgical instruments; as a prophylactic and therapeutic agent in diseases caused by bacteria, viruses and fungi;



for the disinfection of drinking water and swimming pool water and for the sanitization of eating utensils. These uses of iodine will be considered later.

In various combinations, iodine is widely distributed in small quantities in rocks and soils; and despite its low concentrations in sea water, some seaweeds possess the ability to extract iodine and it is found in the ashes when they are incinerated. The Chilean nitrate deposits contain the most important source of iodine. Here it is found as calcium iodate (lautarite) and possibly as one or both of the double salts of sodium iodate with sodium sulfate.

## PROPERTIES OF IODINE

In massive form, iodine, a solid, is bluish-black in color, possessing a specific gravity of 4.63. The material usually seen and official in the U.S.P. and most all official compendia throughout the world is almost opaque, occurring in the form of heavy, grayish-black plates or granules or strongly doubly refracting bluish-black crystals, which have a bright metallic luster and a characteristic odor.

Iodine is the only halogen which is solid at ordinary room temperature (20°C) and it possesses the highest atomic weight (126.91) (Wichers, 1952) of the common halogens. It can change spontaneously into the vapor (nascent) state without first passing through a liquid phase. It melts however at 113.6°C to a black mobile liquid and boils at 184°C under atmospheric pressure to produce the characteristic violet-colored vapor. True liquid iodine is rarely seen except under special laboratory conditions.

Iodine is only slightly soluble in cold water (1 part in 3450 at 20°C), but is much more soluble in boiling water (1 part in 250 at 100°C). It also is readily soluble in aqueous solutions of hydriodic acid and those containing soluble iodides (iodide ion), where polyiodide ions are formed. Iodine is very soluble in organic solvents, where solutions are either violet or brown in color. Chloroform, carbon disulfide, carbon tetrachloride, and benzene solutions produce a violet color, whereas, in alcohol, ether, glycerin, and propylene glycol, a brown color is produced. The solubility of iodine has been reported to increase in the presence of cane sugar (Raikhshtein *et al.*, 1933). For a review of the nature of iodine solutions, see Kleinberg and Davidson (1948). Other solubilities in grams of iodine per 100 grams of solvent are: 0.0340 in water (25°C); 26.6 in ethanol (24.5°C); 1.84 in CCl<sub>4</sub> (25°C); 20.15 in CS<sub>2</sub> (25°C); 16.46 in C<sub>6</sub>H<sub>6</sub> (25°C); 28.91 in ethyl oxide (16.6°C); 1.22 in glycerin, and 0.13 in 50 per cent glycerin. A deep blue color, produced

with starch, disappears when the mixture is boiled and reappears when it is cooled.

*Action of Iodine as a Bactericide.*—Iodine is a highly reactive element and precisely because it is so reactive it is a good germicide. When iodine acts as a disinfectant, free iodine is the effective agent (Nyiri and Dubois, 1931). Sollmann (1948) states that "Elemental iodine precipitates proteins, the iodine being partly absorbed, partly loosely bound, and partly converted into iodide ions. Since the iodine is loosely bound it continues to penetrate so that the action extends deeply." McCulloch (1932) believed that "iodine destroyed microorganisms by the formation of salts with proteins by direct halogenation."

*The Bactericidal Efficiency of Iodine.*—The procedures for testing the efficiencies of antiseptics have been developed and extended since the introduction of the antiseptic era by Lister (1867). In gathering laboratory data on the efficiency of an antiseptic, one must recognize that today there is not as yet available a universal single laboratory test applicable under all conditions. In all instances laboratory data should be obtained and interpreted under conditions paralleling as closely as possible those found in actual practice. Efficacy under conditions of use (as recommended in labeling) is, in the final analysis, the important criterion. When an antiseptic shows constantly satisfactory effectiveness by many different *in vitro* and *in vivo* methods, one can then state that it deserves a high rating of usefulness as a bactericidal agent (Hunter, 1943).

It is obvious that laboratory data are more acceptable as evidence if, in addition to clinical evidence, *in vitro* and *in vivo* laboratory tests have revealed that an antiseptic displayed a constantly satisfactory low toxicity and a high bactericidal efficiency by many different methods.

Iodine has been used as a bactericidal agent for nearly a century and there are many reports by different workers, including those by the author, as to its antisepticity. These reports have all indicated the high bactericidal efficiency and low tissue toxicity possessed by iodine. However, discrepancies exist in the literature as to the highest dilution of free iodine which is effective. This has been due to the results obtained with different non-standardized strains of the same species of microorganisms; to the use of different kinds of bacteria; and the use of different and varying techniques.

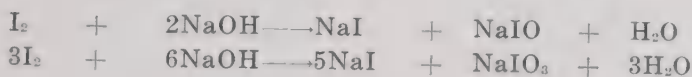
One of the most striking characteristics of iodine, especially noticeable to one who has studied the germicidal efficiencies of the medicinal dyes and the complex mercurials, is that the concentration of iodine necessary to disinfect does not vary greatly with different species of microorganisms (McCulloch, 1945).

In the author's laboratory, where we have checked and rechecked all of the techniques reported, it has been interesting to note the reproduction of the results reported by all authors. We have observed that the bactericidal efficiency of iodine solutions depended upon free iodine, and that an increase in the amount of free iodine within a solution was indicated by an increase in the bactericidal efficiency.

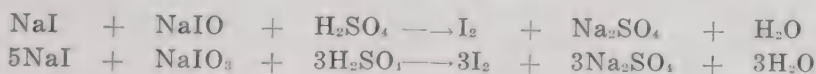
Iodine exerts its effect over a wide pH range, including the reactions apt to be encountered in practical routine. In the author's laboratory, an extensive study revealed interesting data.

The pH values used in our experiments were kept within a range which theoretically gave no germicidal effect *per se* because of alkalinity (sodium hydroxide) or acidity (phosphoric acid). The buffer solutions used to keep the pH values constant did not act as a bactericide.

The reaction between iodine and sodium hydroxide formed sodium iodide, sodium hypiodite, or sodium iodate.



As the alkalinity decreased (acidity increased), more free iodine was liberated. The starting point of this liberation of free iodine, although unknown, occurred at an alkaline pH. In acid solution, the reaction formed more free iodine.



There appeared to be a buffering action in 2 per cent free iodine solutions prepared from Iodine Tincture, U.S.P. XIV, or Iodine Solution, N.F. IX, at the pH range of 6.0 to 7.2 with sterile F.D.A. broth as well as with 24-hour-old cultures of *Micrococcus pyogenes* var. *aureus* in F.D.A. broth. Controls of 2.4 per cent sodium iodide solutions were prepared in buffer solutions. One ml quantities of the 2.4 per cent sodium iodide solutions were added to varying quantities of sterile F.D.A. broth and the pH effect noted. The buffering action within the pH range of 6.0 to 7.2 was identical in the 2.4 per cent sodium iodide solution and in Iodine Solution (N.F. VIII), which consisted of 2 per cent iodine and 2.4 per cent sodium iodide. This indicated that the buffering action of 2 per cent aqueous iodine could be attributed to the 2.4 per cent sodium iodide (Gershenfeld and Witlin, 1949a). The germicidal activity of iodine in the presence of excess potassium iodide does



not vary with pH, since under such circumstances the hypiodous acid (HOI) concentration is negligible even at pH 9.0 (Wyss and Strandskov, 1945).

The phenol coefficient of iodine at 20°C with *Salmonella typhosa* varied from 5 at pH 9.55 to 20 at pH 4.93 (Gershenfeld and Fox, 1948).

Color changes in 2 per cent iodine solutions started at pH 10.9 (10 ml of N/1 sodium hydroxide + 100 ml of 2 per cent iodine). The color, usually considered the end point in the chemical preparation of sodium hypiodite, was attained at pH 11.6 (15 ml of N/1 sodium hydroxide + 100 ml of 2 per cent iodine solution).

The solubility of iodine in 0.1 normal sodium hydroxide was found to be 1.694 per cent of iodine (Gershenfeld and Fox, 1948).

Two per cent free iodine solution (1 ml) was observed to be effective within 1 minute against the following amounts of 24-hour-old F.D.A. broth cultures: 20 ml of *M. pyogenes* var. *aureus*, *S. typhosa*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and 10 ml of *Bacillus mesentericus* (Gershenfeld and Witlin, 1949a).

From the findings it was evident that solutions containing free iodine, 2 per cent, displayed greater bactericidal efficiencies within one minute than was revealed by similar concentrations of free bromine or available chlorine under conditions of the techniques used. Employing the same methods of testing, but adding McIlvaine's buffer solutions, 2 per cent iodine solutions were more effective antibacterial agents within a very wide pH range than were displayed by similar concentrations of the other halogens (Gershenfeld and Witlin, 1949a).

Bactericidal efficiency tests with the dilute halogens (1:5000) revealed that free iodine solutions displayed more effective antibacterial activity against the test bacteria than did chlorine or bromine at 37°C or 24°C either in the presence or in the absence of organic matter. Iodine solutions of such strength were found to be effective for use as antiseptic washes and for irrigation purposes in pH ranges from 2.2 to 8.0 (Gershenfeld and Witlin, 1949 b).

Evaluation of iodine by the Klarmann-Wright technique (Gershenfeld and Palisi, 1949) gave results comparable to those obtained by all other techniques.

Tests including other bactericides (halogens, phenols, mercurials (organic and inorganic), picric acid, the oxidizing agents, dyes, quaternary ammonium compounds, and others) reveal that the results obtained with iodine, employing the varying techniques, are comparable and that iodine deserves a high rating among the most efficient antiseptics in use today.

*Bacteriostasis*.—There has been a clear differentiation made between the properties of killing and inhibiting bacteria (Marshall and Hrenoff, 1937).

Numerous organic and inorganic agents have been reported as being capable of neutralizing the effect of iodine (Kojima, 1940). Among the organic compounds used were serum, glycerin, syrup, feces, ascitic fluid, egg, milk, sputum and urine. Among the inorganic substances were sodium thiosulfate, metallic mercury and ammonia water (Kojima, 1940).

To distinguish between bactericidal and bacteriostatic activity, sodium thioglycollate was added to transplant media for testing antiseptics, but in the case of iodine, no differences in the results were obtained (Hoyt *et al.*, 1942; Gershenfeld and Patterson, 1945). Likewise, sodium thioglycollate, salts of the sulfites and sodium thiosulfate (1 per cent), have been incorporated into media as specific inactivating agents for any iodine which may have been carried over from medication mixtures. Sodium thiosulfate was usually considered because it could be obtained in pure form and in uniform strength each time. The amount of sodium thiosulfate required to react with iodine to produce sodium iodide, sodium tetrathionate and water is based on the molecular equivalents of the chemicals, two molecules of thiosulfate for every molecule of iodine.



In the author's laboratory we have conducted investigations on iodine inactivation in which sodium thiosulfate was incorporated into media and compared with media to which thiosulfate had not been added. We have found that iodine enters into combination with the proteins present in nutrient broth. Because if its protein content, nutrient broth has the ability to inactivate the quantities of free iodine which may be carried over in all of the presently accepted bactericidal efficiency techniques (Gershenfeld and Witlin, 1949a). Results of our studies with iodine inactivation have shown that the dilution of iodine possessing bacteriostatic action and the dilution of iodine having bactericidal action were practically identical.

We have previously reported the intensity and rapidity of the action of iodine in experiments demonstrating the relationship between the killing and stasis of tissue *in vitro* by antiseptics in concentrations used in practice (Witlin, 1941a). Here again there is evidence that iodine, with a toxicity lower than phenol, kills immediately rather than by a prolonged period of stasis. This was later confirmed by Salle and Catlin (1947) who observed that the

effective concentrations of iodine represented true killing values and that no bacteriostasis was involved. They also reported that the highest dilution of iodine killing within 1 minute (1:20,000) was the same as for 10 minutes, a property not shared with any of the other antiseptics.

Since the effectiveness of an antiseptic may be altered in the presence of proteins or other organic matter, *in vitro* tests are frequently conducted in the presence of organic matter. This is usually accomplished by the addition of serum (human, horse or other animal) before the addition of the broth culture of the test organism. Some workers have employed organic matter other than serum (Mallmann and Chandler, 1933; Ruehle and Brewer, 1931). The relationship of the interaction of iodine with blood and its constituents was reported by our laboratory in 1932 (Gershenfeld and Miller, 1932) and studied in greater detail by us in 1947 (Gershenfeld and Witlin, 1948a).

*Toxicity tests.*—Various toxicity tests have been introduced as aids in determining the toxic effects of antiseptics on different kinds of living tissue, as contrasted with their lethal effect on microorganisms. All of the tests should be interpretable in terms of practical use. It is obvious, therefore, that laboratory data will be more acceptable if, in addition to the commonly employed bactericidal efficiency tests, other *in vitro* and *in vivo* tests be conducted. The most desirable antiseptic should possess a high bactericidal efficiency with low toxicity by many different methods. This has been accomplished by exposing various tissues, both *in vitro* and *in vivo*, and a culture of microorganisms in an antiseptic and comparing inhibition of tissue activity with germicidal action.

Iodine has consistently been reported upon favorably in *in vitro* and *in vivo* toxicity tests.

Tissue culture techniques have been employed for the evaluation of iodine employing human skin (Lambert and Meyer, 1926), embryonic chick heart fragments (Salle *et al.*, 1938; Witlin, 1941b; Paff *et al.*, 1945), frog tissue (Samuels, 1938), and rabbit spleen (Lambert and Meyer, 1926). Penetration, injury and skin sterilization have been conducted in *in vitro* and *in vivo* tests on the skin of humans (Novak and Hall, 1939; Colebrook, 1941; Neate, 1911; Bost, 1944; Gardner, 1948), horses (Kelser and Mohri, 1932), rabbits (Bass, 1939; Gershenfeld and Witlin, 1941) and other animals (Sarber, 1942a, b; Tiner *et al.*, 1946). The effect of iodine has been studied on the mucous membranes (Sarber, 1942a, b; Miller and Appleton, 1931; Meyer and Arnold, 1938), root canals of the teeth (Grossman, 1946; Seltzer and Bender, 1949) and upon open wounds (Simmons, 1933; Babcock, 1937, 1948; Skvortsov, 1938). Tincture



of iodine has been used full strength on the human peritoneum (Strong, 1930). The eye has been used for observation of ocular irritation and lysozyme inhibition (Thompson *et al.*, 1937). One technique (Nye, 1937) combined germicidal efficiency tests, diffusion through paraloidion sac procedures, leukocytic toxicity determinations, and toxicity observations by the intradermal injection of the substances under test. Cutaneous lesions produced in mice with virulent staphylococci have been topically treated with antiseptics for evaluation purposes (Hunt, 1937).

Infection-prevention techniques employing mouse tails (Nunnester and Kempf, 1942; Gershenfeld and Miller, 1947) and abdominal tissue (Sarber, 1942 *a, b*) have been devised as a means of determining the value of iodine and other substances as antiseptics in terms of their action on the skin as well as their effectiveness in preventing infection. A comparison of the percentage of mouse survivals and fatalities was used as the index of effectiveness.

The egg injection technique for toxicity was devised in our laboratory (Witlin, 1942) and a comparison of the antiseptic's toxicity for chick embryos with its bactericidal efficiency determined (Gershenfeld and Witlin, 1947). A modified technique of infecting the membranes of the embryo and then administering the antiseptic has been developed (Green and Birkeland, 1942, 1944).

Manometric observations of the effect of iodine and other substances on microorganisms have been compared with mouse minimum lethal dosage of iodine (Bronfenbrenner *et al.*, 1939).

Iodine and other substances have also been evaluated by comparing their bactericidal efficiency with their ability to destroy the osmotic complement contained in human and animal blood, as measured by the white blood cells being unable to engulf infectious bacteria (phagocytosis) (Welch *et al.*, 1939, 1940, 1942).

In all of the *in vitro* and *in vivo* evaluations, iodine has been shown to be very high in germicidal efficiency and relatively low toxicity.

*Penetration.*—Claudius (1902) while searching for a method of sterilizing catgut found that iodine had penetrability as demonstrated by the death of microorganisms enclosed in the minute folds of tissue. Nine years later, to demonstrate the penetrability of iodine, Kendall and Edwards (1911) employed uniform cylinders of seeded agar, and Seelig and Gould (1911) used collodion capsules with living animal skin. Carnot and Dumont (1918) used seeded agar plates containing centrally embedded perforated glass disks to determine the penetrative powers of iodine in wound dressings.

Knaysi (1930 *a, b, c*) presented an extensive review on penetration of the cell wall and the mode of action of bactericides and Knaysi and Gordon (1930 *a, b*) demonstrated iodine's ability to penetrate the yeast cell.

Karns (1932) and Karns, Cretcher and Beal (1932) reported the penetration of iodine-iodide solutions in silk fabric and guinea pig skin. Biskind (1932) demonstrated the rate of speed of penetration of aqueous iodine through frog skin. Nyiri and Jannitti (1932) studied the action of iodine on the unbroken skin of dogs and rabbits and found that iodine only in the form of its compounds such as potassium iodide penetrated the live skin within 162 hours. Von Oettingen *et al.* (1932) reported the penetration of iodine into leather. Mallmann and Chandler (1933) found that, of a group of recognized disinfectants, only colloidal iodine was able to penetrate and destroy the bacteria embedded in finely divided particles of avian fecal matter. Anderson and Mallmann (1943) reported the superior penetrative power of iodine using the wall of a chicken intestine, rabbit skin, coccidial oocysts and strongyloid larvae.

*Effect on Tubercle Bacilli.*—The statements that "tubercle bacilli are resistant to the usual chemical disinfectants" and that they are resistant to the action of chlorine are common. Data of recent origin concerning the tuberculocidal action of free iodine is not extensive. Knaysi (1932) noted that tubercle bacilli did not grow on slants after 30 minutes' contact with dilutions of iodine as high as 1:10<sup>4</sup> nor did guinea pigs contract the infection after injections of a mixture of the organisms exposed for 15 minutes in a dilution of 1:5 x 10<sup>3</sup>. Hailer (1938) tested various chemicals for their tuberculocidal activity and among those proven to be effective by his *in vivo* technique was tincture of iodine (German Pharmacopeia formula). Sommermeyer and Frobisher (1952) in a study on the disinfection of oral clinical thermometers used thermometers contaminated with sputum which was "heavily positive for acid-fast bacilli on direct smear examination." They found that "aqueous solutions of 0.05 per cent and 0.25 per cent iodine in 1 per cent potassium iodide when used as disinfectants in this study yielded surprisingly small numbers of positive cultures. Time did not permit investigation of higher concentrations of iodine in aqueous solutions after it was recognized that these might be good disinfectants."

In the author's laboratory (Gershenfeld *et al.*, *in press*) the effect of iodine on *Mycobacterium tuberculosis* var. *hominis* (Gs) was noted in three different kinds of investigation, growth, respiratory and animal studies. Under the conditions of these experiments, concentrations of free iodine from 0.0625 per cent and

stronger were tuberculocidal in a 100 mg/ml strength after a 5 minute exposure at room temperature. Using the Warburg respirometer, respiration of the strain of tubercle bacillus used was inhibited as follows: 0.05 per cent exhibited 87 to 100 per cent inhibition; 0.005 per cent exhibited 78 to 96 per cent; and 0.0005 per cent exhibited 0 to 7 per cent inhibition. In the *in vivo* experiments, clinical symptoms of tuberculosis were entirely absent in guinea pigs injected with *M. tuberculosis* var. *hominis* (Gs), 10 mg/ml exposed to 0.5 and 0.05 per cent free iodine. Even a strength of 0.005 per cent free iodine markedly reduced the virulence of this strain.

*Sporicidal Efficiency.*—Iodine has been reported to possess sporicidal efficiency. Kelser and Mohri (1932) reported that 1 part of iodine killed 10 parts of suspension of tetanus spores within 10 minutes. In an investigation by Hailer and Von Bockelberg (1939) on the toxic effect of halogens using dried anthrax spores, iodine was found to be the most effective. The sporicidal action of iodine at pH values ordinarily encountered is due primarily to the free iodine, although at high pH values hypiodous acid may also exert some action (Wyss and Strandkov, 1945).

In recent study in the author's laboratory (Gershenfeld and Vitlin, 1952), the following conclusions were reached on: "the effect of Iodine Solution N. F. IX (pH 4.6) upon one year old spores of *B. anthracis*, *B. subtilis*, *B. megatherium*, *B. mesentericus*, and *Cl. tetani* by both 'wet' and 'dry' techniques. The same procedures were used employing lower pH values (4.0, 3.0, 2.0, and 1.0).

"The results observed in this series of experiments revealed that:

- "1. Two per cent aqueous iodine (Iodine Solution N.F. IX) possesses sporicidal efficiency.
- "2. The efficiency of Iodine Solution N.F. IX against the spores of *B. subtilis*, *B. anthracis*, *B. mesentericus*, *B. megatherium*, and *Cl. tetani* was enhanced at lower pH values (4.0, 3.0, 2.0, and 1.0). Controls of hydrochloric acid in water at the same pH values were ineffective against all of the test spores under the conditions of the procedures used in this study.
- "3. Wet suspensions of spores on an absorbable surface (filter paper) required longer periods of exposure than those on non-absorbable surfaces (razor blades) before being killed.
- "4. Spores dried on an absorbable surface (filter paper) required shorter periods of exposure than those on non-absorbable surfaces (razor blades) before being killed."



Because of its rapid and high bactericidal efficiency, iodine has been recommended as an "emergency sterilizing agent" for surgical instruments (Hoyt *et al.*, 1942).

*Fungicidal Activity.*—Iodine has been found to possess both a high fungicidal and fungistatic efficiency (Hoffman *et al.*, 1941; Schmidt, 1940) against *Sclerotinia fructicola*, *Alternaria solani*, *Penicillium expansum* and *Rhizopus nigricans* (McCallan and Wellman, 1942).

Seltzer and Bender (1949) reported iodine as being fungistatic at 0.01 per cent and fungicidal at 0.1 per cent against *Monilia albicans*.

Iodine has been reported to possess fungicidal efficiency for *Trichophyton gypseum* and *Monilia albicans* in a concentration of approximately 1:3000 (Emmons, 1933). Iodine in a 1:85,000 dilution has been most effective against *Epidermophyton inguinale* after 15 minutes exposure (Schamberg *et al.*, 1931).

Species of *Monilia*, *Torula*, *Epidermophyton* and *Trichophyton* required an iodine concentration of 1:715 for fungicidal action, whereas species of *Saccharomyces* required a 1:1430 dilution (Gomez-Vega, 1935). Strickler (1932, 1946) reported the effective fungicidal properties of vaporized iodine on human skin diseases and of a special iodine preparation for ringworm of the scalp. Vapors of iodine destroyed cultures of *Trichophyton tonsurans* within five minutes (Zifferblatt and Seelaus, 1933).

In a recent study on the fungicidal action of various agents on scalp hairs infected with *Microsporum audouini*, 2 to 5 per cent tincture of iodine was found to be definitely fungicidal (Monash, 1952).

Fruit has been wrapped in iodine-impregnated paper to retard the growth of mold responsible for destroying the fruit.

In a review of the literature on the use of organic compounds of iodine against insects, fungi and bacteria, compiled by Bowen (1944) of the U. S. Department of Agriculture, 294 organic iodine compounds are listed, of which 112 were tested as insecticides or fungicides. Swingle *et al.* (1944) studied the toxic effects of 883 organic compounds on different insects and classified them on the basis of toxicity. Thirty-four of these compounds contained iodine. Only 22 per cent of the total number of compounds tested were found to be effective, yet 55 per cent of those containing iodine were in this group.

*Effect of Iodine on Viruses.*—Iodine has been demonstrated to possess virucidal activity. Tincture of Iodine U.S.P. XII, diluted to contain 0.1 per cent free iodine, and Lugol's Solution U.S.P. XII, diluted to contain 1 per cent free iodine, were effective in inactivat-

ing vaccinia virus CAEB within 3 minutes (Dunham and MacNeal, 1943). For its effect upon poliomyelitis virus, see page 190.

Iodine has been used prophylactically with success against influenza (Dunham and MacNeal, 1944) and herpes (Hruszek, 1934) and therapeutically in cases of variola and varicella (Boudreau, 1916). Little activity was displayed by iodine against the virus of tobacco mosaic (Anson and Stanley, 1941).

Kaiser (1939) suspended various types of virus in water and aerated them with air which had first been passed through a 10 per cent alcoholic solution of iodine. By so doing, vaccinia virus was inactivated in thirty seconds and could not be reactivated. By suspending the virus directly in a series of dilute solutions of iodine, the minimum concentration of iodine for inactivation was found to be 1 : 1,000,000. Similar results were obtained with myxoma virus.

## USES OF IODINE

*Two per cent Iodine Solutions.*—The 2 per cent (w/v) Iodine Tincture (U.S.P. XIV) and Iodine Solution (N.F. IX) are the iodine preparations of choice for use as antiseptics.

The author has recommended the aqueous iodine solution in preference to the tincture for reasons stated later and presented in more detail in articles published by him.

Too often preparations labeled "Iodine Tincture" are used which contain quantities of free iodine or iodide or alcohol other than those present in the U.S.P. preparation. This labeling is not only illegal but objectionable for many reasons. It therefore is desirable to clarify the Iodine Tincture issue, as confusion still prevails.

Today and since 1947 on the market in this country, Iodine Tincture refers to one preparation and one only, the U.S.P. tincture containing 2 per cent iodine, 2.4 per cent sodium iodide in diluted alcohol (the final alcohol content is 47 (44 to 50) per cent). Unfortunately, prior to 1947, the name "Tincture of Iodine" referred to the then U.S.P. preparation (now official in the N.F. as Strong Iodine Tincture) but which differs in composition from the present U.S.P. preparation now known as "Iodine Tincture." The U.S.P. preparation official prior to 1947 as Tincture of Iodine contains 7 per cent iodine, 5 per cent potassium iodide in an alcoholic solution (final alcohol content from 83 to 88 per cent). The Tincture of Iodine official during still earlier years contained even a higher alcohol content. The alcoholic 7 per cent free iodine preparation is excessively strong and may be irritating when used on the skin and covered tightly. This strong tincture is not generally rec-

commended for use as an antiseptic, but is primarily employed as a counterirritant. Unfortunately even today the U.S.P. Iodine Tincture, containing 2 per cent iodine, 2.4 per cent sodium iodide in diluted alcohol, is not always used as the antiseptic alcoholic solution. One erroneously labeled iodine tincture is frequently supplied which contains either a stronger iodine or alcohol (or both) content; also the iodine-iodide ratio may be different. Furthermore iodine tincture, reported in the American literature prior to 1947 and found at anytime even today in foreign literature under this name usually refers to a preparation which may be different in composition from Iodine Tincture, U.S.P. XIV. The iodine tincture situation even in its manufacture has had its shortcomings, as reported in an editorial by Tice (1947). Recently the iodine-iodide solubility relation in water was established for a limited range at different temperatures (Goldstein, 1952).

*Iodine Solution, N.F.*—A water solution of iodine-iodide (Iodine Solution, N.F.) is preferred for most uses. Such a solution avoids the possibility of most of the objections to alcoholic solutions. This water solution of iodine is ideal as an antiseptic for a variety of uses. More than twenty years ago, after extensive work in the author's laboratory, we reported (Gershenfeld and Miller, 1932) that a water solution of iodine "containing soluble iodides is more satisfactory as an antiseptic than most of all the other commonly employed marketable antiseptic preparations." In a report on antiseptics for hospital use (Palmer, 1948), the statement is made: "in dilute aqueous solutions, iodine remains one of the most effective chemical germicides available." Homans (1948) states: "Watery solutions of iodine are superior germicides," indicating that weak dilutions are "not only able to check the growth of bacteria but to kill them, and that in the presence of blood serum." Furthermore, "Its usefulness as a rinsing solution (in the operating room) and as an antiseptic irrigation fluid for both potentially and grossly infected wounds has probably been overlooked." Ficarra (1951) refers to the use of both the N.F. Iodine Solution and iodine-propylene glycol solutions. Aqueous solutions of antiseptics, during cold weather or in cold climates, if in glass containers, may freeze and break. As noted by the authors (Gershenfeld and Witlin, 1950 *a, b*), if an aqueous solution must be shipped during cold weather: "Propylene glycol can be employed to lower the freezing point of aqueous solutions of iodine. Propylene glycol-iodine solutions possess an adhesiveness on human skin which may be desirable."

*Iodine as a Skin Antiseptic.*—Iodine has been widely used as an antiseptic for the preoperative preparation of the skin and for the treatment of various skin affections.



It is recognized today that even the normal skin is never free of microorganisms, both transient and resident. The latter, the normal inhabitants, are probably constant in numbers and are located in the deeper parts of the skin, especially in the hair follicles and the sebaceous glands. The transient flora, found on the superficial skin surface, vary in numbers depending upon cleanliness and contact contamination of the individual and his environment. The resident flora are difficult to remove; however, unless large numbers are carried along, they rarely are responsible for serious wound or skin infections. The transient flora, usually responsible for most infections, can be removed by mechanical and chemical cleansing of the skin. In the preoperative preparation of the skin, mechanical cleansing by proper vigorous scrubbing wherever possible will remove most of the transient and a high percentage of the resident flora. But as indicated by Walter (1948): "Chemical disinfectants must be relied upon to destroy more of the organisms remaining on the clean, dry, fat-free skin." Lovell (1946) advises that: "Chemical cleansing should be performed to further reduce the number of bacteria on the surface. Numerous good antiseptics are available but 3.5 per cent iodine is probably used more frequently than any other chemical on the patient's skin preparatory to the operation." Kulaski (1947) states that: "Soaps should not be relied upon to produce skin sterility. After mechanical cleansing it is necessary to eliminate the transient and resident bacteria. Iodine has a wide reputation as an efficient germicide . . . Although the tincture has been so widely used, the aqueous solutions of iodine are gaining in prominence as more and more reports appear on their highly germicidal action . . . For preoperative skin preparation the aqueous solutions possess certain advantages."

Dannreuther (1908) indicated the following: "I do not believe that the value of iodine in surgery is as generally appreciated as it deserves." Noehren (1947), describing many years of experience with thyroidectomy, states: "The operative area is wiped with ether to remove all fat and sebaceous material from the skin and this is followed by a not too wet application of half strength tincture of iodine. We have never seen any reason for changing to any other antiseptic."

Blakemore (1948) discusses the routine established at the Presbyterian Hospital in New York City for the treatment of aneurysms. Included is "skin preparation by a soap and water scrub followed by alcohol, ether, iodine and alcohol." Babcock and Bacon (1945), in discussing the surgical treatment of carcinoma of the large bowel, "prefer a 3.5 per cent tincture of iodine." Horsley and Michaux (1946) prepared the skin of the abdomen by treatment with an iodine-alcohol solution.

Veenboer and Kooistra (1947), in operations showing third degree uterine prolapse, swab the vagina with a 2 per cent iodine solution followed with 70 per cent alcohol, and Roman (1946) mentions that in his operative technique he employs "3.5 per cent iodine and alcohol." Chace (1948) likewise indicated that in cataract operations "Iodine, 3 $\frac{1}{2}$  per cent, is painted on the skin of the lids and cheek and wiped off with 70 per cent alcohol."

Picchioni (1942), in a review on skin disinfection, states, "of the various germicidal agents included in this review, it is concluded that a solution of iodine is a most satisfactory preparation for preoperative skin preparation." Gardner's findings (Lancet, 1952) are reported on "a wide range of antiseptics, measuring the ability of a single application to achieve virtual disinfection of a standard area of skin on which a suspension of *Micrococcus pyogenes* var. *albus* (or certain other organisms, including *P. aeruginosa*) had been swabbed and allowed to dry. The only solution that did this regularly in 15 to 20 seconds was 2 per cent iodine in 70 per cent alcohol." These statements by Walter (1948) are of interest: "One of the most striking characteristics of iodine as a germicide is its uniformity of action on different species of microorganisms . . . Aqueous solutions are more effective germicides, do not irritate the tissues, and are painless."

Finally, attention is directed to a recent Annotation (1952) on "Skin Disinfection" which in commenting on Story's (1952) studies states "The results confirm in the first place an observation on which almost all reliable workers are unanimous—namely, that tincture of iodine is highly and rapidly effective." Story (1952) presents a new method of testing skin disinfectants and reports that "1 per cent iodine in 70 per cent industrial spirit (74 O.P.) killed all test organisms within 30 seconds" and 1 per cent aqueous iodine was "effective within 30 seconds against the bacteria tested."

*Technique of Preoperative Preparation of Skin.*—In preparing the field of operation, a generous area, determined separately for each operation, is included surrounding the proposed incision. Just before the drapes are applied, rub or swab vigorously the 2 per cent iodine solution into the skin preferably for 2 minutes (not less than 1 minute). If rubbing is not advisable, the iodine solution should be kept moist on the operative site preferably for 5 minutes or used for this period of time as a wet dressing. If desired the excess moisture can be wiped with 70 per cent alcohol or a sterile solution of sodium thiosulfate. Where there is no objection to the iodine layer, it need not be removed but employed as suggested by Ficarra (1951).

*Iodine for Other Skin Uses.*—Rigid aseptic precautions are required for obtaining blood. Iodine Tincture or Solution is the ideal, safe antiseptic for the disinfection of the skin area prior to the removal of blood for transfusions and blood banks (Walter, 1948; Braude *et al.*, 1952) or for testing purposes; when removing spinal fluid and transudates such as pleural, peritoneal and synovial effusions; also when obtaining bone marrow by vertebral spinous process puncture (Memorandum, 1947; Huss *et al.*, 1948) or when cutting a skin graft (Webster, 1945). The same rigid aseptic precautions and the use of Iodine Tincture or Iodine Solution should be employed when administering medicaments parenterally, especially intravenously, intrathecally and intramuscularly. Injections given subcutaneously or intracutaneously or tests performed by either route are frequently administered only after wiping the skin with alcohol. This procedure as usually carried out, though satisfactory in many instances, is not as safe as when using Iodine Tincture or Iodine Solution.

Iodine solutions have been and are used for the disinfection of hands of surgical teams and for the treatment of wounds. For details of the use of 1:1000 and weaker solutions of iodine as antiseptic washes, see Ficarra (1953).

*Iodine for Disinfection of Instruments and Other Equipment.*—Iodine has been recommended as an "emergency sterilizing agent" for certain equipment because of its "rapid and high bactericidal efficiency," and this is especially so in those instances where heat may affect the equipment. This may include the disinfection of catheters, knife blades, plastic items, rubber goods, brushes, ampuls, and multiple-dose vials. Iodine solutions in strengths from 0.5 to 2 per cent (of free iodine) can be used for the disinfection and as a storage liquid for brushes in the operating room. In the terminal disinfection of materials after operation, these can be placed in a container in which an iodine solution is present until they are later sterilized in the autoclave.

*Catgut and Other Surgical Suture Materials.*—The nonboilable or hydrated type of surgical gut must not be heated and it is disinfected by a suitable antiseptic solution. The hydrolysis caused by the heat will destroy the gut. In case of the boilable or anhydrous type or other suture materials which can withstand steam sterilization, the latter procedure may not be available or is not adequate for the sterilization of enormous quantities as may be needed at times. Also the exterior of the unopened suture tubes must be disinfected before use. The tubing fluid used today by some manufacturers contains iodine or iodine compounds as the antibacterial agent.



With the wide use of all kinds of textile and synthetic fibers, it is not possible to make a general statement that all of them can be disinfected by one chemical solution. In occasional instances, it is possible that the agent used may affect the tensile strength. Such information can only be obtained after trying the chemical and the technique advocated.

The very nature of surgical sutures accounts for the fact that they are contaminated and frequently this makes it difficult to effect positive sterilization. Also the use for which they are employed makes it imperative that they be properly sterilized. Since 1902, catgut for surgical sutures has been reported as having been "sterilized with iodine." Christensen (1944), in discussing catgut sterilization, recommends the use of a 1 per cent iodine solution. He indicates that even after 4 hours exposure, there was but little effect upon the tensile strength, and the treated material was "absorbed exactly as catgut manufactured in other ways." Catgut for surgical sutures has been reported by others as having been "sterilized" with iodine (Kellaway and Williams, 1934; Fandre, 1944; Price *et al.*, 1946; Holder, 1946).

*Disinfection of Clinical Thermometers.*—In the author's laboratory (Gershenfeld *et al.*, 1951), the antibacterial efficiency of various commonly used solutions for the cold disinfection of clinical thermometers was determined. "The compounds used were iodine solutions (2 per cent) N.F. IX, iodine tincture (2 per cent) U.S.P. XIV, ethyl alcohol (95 per cent), ethyl alcohol (70 per cent by weight), ethyl alcohol (50 per cent by volume), isopropyl alcohol (70 per cent by volume) and isopropyl alcohol (50 per cent by volume).

"Graduated clinical thermometers which were cut into sections two inches long and sealed at both ends were used. The sterile sections were treated with cultures of test bacteria. The test organisms used in the study were *Diplococcus pneumoniae*, *M. pyogenes* var. *aureus*, *Streptococcus hemolyticus*, *Streptococcus fecalis*, and *E. coli*. The test organisms were thoroughly dried onto the thermometer sections. The treated thermometer sections remained in the disinfectant solution at 20°C for the designated time period, were rinsed for 10 seconds in sterile distilled water, and transferred to tubes containing media. Thermometer sections treated with iodine solutions were transferred after treatment to media containing 1 per cent thiosulfate. The transplant tubes were incubated for 48 hours at 37°C, and the cultures were examined for observation of growth.

"In order to determine the effect of large quantities of organic matter upon the disinfectants under test, citrated human blood

plasma in concentrations of 25 and 50 per cent, was added to the initial culture media tubes containing a 24-hour growth of *M. pyogenes* var. *aureus*.

"1. In tests conducted against *S. hemolyticus* the iodine (tincture 2 per cent and solution 2 per cent) and alcohols, with the exception of ethyl alcohol 95 per cent, killed the test organisms within 20 seconds.

"2. Iodine tincture 2 per cent (U.S.P. XIV) and iodine solution 2 per cent (N.F. IX) killed *S. fecalis* within 100 seconds, while the alcohol disinfectants did not kill within 120 seconds.

"3. The iodine preparations killed *E. coli* more quickly than did the alcohols, while ethyl alcohol 95 per cent did not kill within 20 seconds.

"4. Iodine tincture (2 per cent) killed *M. pyogenes* var. *aureus* within 80 seconds, while iodine solution 2 per cent required 120 seconds to kill. Ethyl alcohol 95 per cent was ineffective against *M. pyogenes* var. *aureus* within 10 minutes. The most efficient alcohol was isopropyl 70 per cent, which killed *M. pyogenes* var. *aureus* within 4 minutes, while the other alcohol disinfectants killed within 10 minutes.

"5. Tests conducted with a 50 per cent plasma—*M. pyogenes* var. *aureus* culture mixture revealed that iodine solution 2 per cent killed *M. pyogenes* var. *aureus* within 5 minutes. The other test disinfectants did not kill the test organism within 10 minutes.

"6. In similar tests using a 25 per cent plasma-*M. pyogenes* var. *aureus* culture mixture, iodine solution (2 per cent) killed *M. pyogenes* var. *aureus* within 3 minutes, while the other test disinfectants were ineffective within a 10-minute test period.

"7. Iodine tincture U.S.P. or iodine solution N.F., widely used antiseptics which are readily available everywhere, were found to be more effective than either ethyl alcohol or isopropyl alcohol."

Other studies (Sommermeyer and Frobisher, 1952; Sommermeyer and Carroll, 1952) on the disinfection of mouth thermometers, using tuberculous sputa and pathogens of the respiratory tract as the contaminating substances, showed that, when "immersion of contaminated thermometers in 0.5 to 1 per cent solutions of iodine in either 70 per cent ethyl alcohol or 70 per cent isopropyl alcohol for 10 minutes" is preceded by an effective cleaning procedure, "the probability that viable bacterial pathogens of the respiratory tract will remain on the thermometer is reduced to a very low level."

*Disinfection of Water.*—In "Advances in Military Medicine" (Committee on Med. Research, 1948), water disinfection, especially

in the field, is detailed. Iodine, 8 ppm, for a contact time of 10 minutes was found to kill water-borne pathogens including amebic cysts, the cercariae of the schistosomes and the leptospirae of infectious jaundice. In this dosage, "high-level intake of iodine-treated water by men undergoing physical exertion under simulated tropical conditions produced no noticeable chemical disturbances, metabolic changes or interference with work performance." For canteen use, a tablet was made available containing tetraglycine hydroperiodide. One tablet is used for each canteen for a 10 minute period. Two tablets for 10 minutes are required for highly colored waters. For cold water, the time period is extended to 20 minutes.

In practical studies at Harvard University, workers (Chang and Morris, 1952) have found: "that iodine residuals of 3 to 4 ppm consistently reduce  $10^6$  bacteria per ml to less than 10 per 100 ml within 10 minutes at  $25^{\circ}\text{C}$  and 20 minutes at  $3^{\circ}\text{C}$ . The organisms, *E. coli*, *S. typhosa*, *S. schottmülleri*, and *Sh. dysenteriae*, as well as the mixed coli-aerogenes flora of sewage, exhibit similar results, while *Vibrio comma* is somewhat more readily destroyed.

"Cysts of *Entamoeba histolytica* are more resistant to iodine than are the enteric bacteria.

"*Leptospira icterohemorrhagiae* is very sensitive to iodine, less than 1 ppm being required to destroy activity of the organisms within 5 minutes at room temperature. Poliomyelitis virus appears to be more resistant, but inactivation has been observed with iodine residuals of a few parts per million and contact times of 5 to 10 minutes.

"It is concluded that iodine is an excellent disinfectant for water supplies under emergency conditions or in other circumstances.

"It is particularly useful when waters of highly variable quality may be encountered and when no treatment other than disinfection is possible. For such applications a minimum iodine residual of 5 to 6 ppm is recommended with a contact time of 10 minutes at  $20^{\circ}$  to  $25^{\circ}\text{C}$  and 20 minutes near  $0^{\circ}\text{C}$ ."

Other workers (Morris *et al.*, 1952) at the same institution reported that: "Of the various possible types of iodine-releasing materials, the periodides have been shown to be preferable for use in single tablets designed for batch treatment of small quantities of water. A number of periodides possessing the requisites of stability and ease of solubility have been prepared, of which one, tetraglycine hydroperiodide, has been adopted as a disinfectant for water in canteens by the U. S. Armed Services. Studies on tablets containing approximately 20 mg of tetraglycine hydroperiodide, 90 mg of disodium dihydrogen pyrophosphate, and 5 mg



of talc show that this composition has good stability for extended periods of storage at elevated temperatures when suitably packaged." For further details concerning the individual iodine water purification tablets of water in canteens, see Military Specification (1952).

Chambers *et al.* (1952) reported recently on the bactericidal efficiency of iodine paralleling conditions in water works and swimming pool practice, noting conditions influenced by exposure time, concentration, pH and temperature. "Studies included one or more species of *Escherichia*, *Aerobacter*, *Salmonella*, *Shigella* and *Streptococcus*." They found that "the minimum average iodine concentration which kills all tested species in one minute under the adverse conditions of pH (9.15) and temperature ( $2^{\circ}$  to  $5^{\circ}\text{C}$ ) is 4.3 ppm" and "The minimum average iodine concentration which kills all tested species in one minute under the most favorable conditions of pH (6.5) and temperature ( $20^{\circ}$  to  $26^{\circ}\text{C}$ ), is 0.6 ppm."

Previously the United States Public Health Service (Hitchens, 1922) recommended the disinfection of drinking water by the addition of the 7 per cent tincture of iodine in an amount approximately equivalent to 1 drop of the latter per quart of water and a time period of from 10 to 30 minutes depending upon the clarity. This gives a weaker solution of free iodine than the above mentioned ppm. Six minims of the 2 per cent iodine solution or tincture per quart yield approximately 8 ppm.

In times of emergencies when drinking water is at a premium, the above treatment will assure a safe potable water for human consumption. Where a sterile water is needed and heating methods are not available, the free iodine content can be increased.

*Sanitizing Eating and Drinking Utensils.*—It is of even greater importance to properly treat eating and drinking utensils, especially if they are used by the sick. Iodine will serve as a useful sanitizing agent for such purpose. In a detailed study conducted in our laboratory, we (Gershenfeld and Witlin, 1951) found that: dishes, cups, glassware, knives, forks and spoons which had been soiled and given a preliminary 10 second rinse in water (between  $100^{\circ}$  and  $170^{\circ}\text{F}$ ) were sanitized by a one-second immersion in iodine solutions (100 and 200 ppm).

"The dilutions of free iodine tested ranged from 200 ppm to 0.5 ppm. Solutions containing free iodine were prepared from Iodine Solution (2 per cent) N.F. IX. The 200 ppm free iodine solution was prepared by placing 1 ml of the 2 per cent iodine solution in 99 ml of distilled water." We found that a concentration of 200 ppm "gives greater safety throughout the 8-hour working day. One teaspoonful of Iodine Solution (2 per cent) (N.F. IX) or Iodine

Tincture (2 per cent) (U.S.P. XIV) to a pint of water will give a solution of approximately this strength. Solutions of this concentration were found to have no adverse effect upon any kind of utensils after repeated treatments.

"It is possible to quickly prepare free iodine solutions containing 200 ppm for use everywhere including its use in the home when sanitization is to be practiced for eating utensils used by the sick.

"Solutions of free iodine of sufficient strength to be used safely in food utensil sanitization were observed to range in color from yellow to deep amber, while solutions too dilute to inhibit the growth of the test organism were very pale yellow. This change in color can be used to advantage in judging whether solutions of free iodine still possess their sanitizing strength."

*Disinfection of the Air.*—It has again been indicated that the transmission of disease takes place via the aerial route (Medical Research Council War Memorandum No. 11, 1944). Plesch (1941) recommended the aerial disinfection of air-raid shelters with iodine as a prophylactic measure against influenza.

Lombardo (1926) advocated the use of iodine as an aerial disinfectant but gave inconclusive evidence for acceptance at that time. During an influenza epidemic, Lombardo diffused iodine vapor into the air of one classroom and after fourteen days found that there had been no cases of influenza in the iodine-treated class whereas the number of absentees was noticeable in untreated classes.

Lipkin (1934) and Lebduska and Pidra (1940) tested iodine vapors for their effect against bacteria at varying distances and iodine was reported to head the list of 128 chemical substances tested for germicidal efficiency by this method.

In 1944, iodine was reported to be effective as an aerial disinfectant at concentrations much below its saturation vapor pressure in air (White *et al.*, 1944). Iodine at a readily tolerable concentration of 0.1 mg/ft<sup>3</sup> of air has been found to be an effective aerial disinfectant, rapidly killing freshly sprayed salivary organisms at humidities above 50 per cent (Raymond, 1946).

*Detection of Free Iodine.*—The classical starch solution, yielding a blue color, is not a suitable reagent for detecting minute quantities (ppm) of free iodine. Studies in the author's laboratory (Gershenfeld and Witlin, 1949c) revealed that *α-naphthylflavone* was "a very sensitive reagent or indicator to detect free iodine in amounts of less than 1 ppm."

## OTHER PREPARATIONS CONTAINING FREE IODINE

There are many preparations marketed under different trade names which are either proprietary brands of iodine tincture or iodine solution or varying concentrations of the latter. "Bryant's Sherry," named after the well-known London surgeon, William Bryant, and widely used more than a half century ago, was a weak solution of free iodine.

Pregl's Solution (Septoiod), containing iodide, hypoiodite, and iodate ions, together with free iodine (0.035 to 0.04 per cent), is employed as an antiseptic isotonic solution.

Karn's Iodine Solution (Karns, 1932), containing 1 part iodine, 0.044 parts of calcium iodide hexahydrate, 0.048 part of potassium iodide, and 1.104 parts of sodium iodide, or prepared in any desired strength of free iodine using the ingredients in the above ratio was at one time widely advocated as an isotonic iodine solution. It also is available on the market as "Isodine," though the latter may be found to contain some glycerin or propylene glycol to prevent freezing.

Colloidal iodine solution, prepared electrically and elemental iodine powder in a colloidal state, stabilized with a suitable protecting colloid, have been recommended as antiseptics. "Medine" has been introduced recently as "specially processed elemental iodine dispersed in an inert bland base in the form of a colloidal sol or emulsion." The iodine concentrations are from 1 to 2 per cent depending upon the specific marketable form.

Strong Iodine Solution, U.S.P. XIV, Strong Iodine Tincture, U.S.P. IX, Churchill's Tincture of Iodine (Iodine, 16.5 g; potassium iodide, 3.5 g; water, 25 cc; and alcohol to make 100 cc) and similar preparations containing high concentrations of free iodine, though used for other therapeutic purposes, are antiseptics and are frequently employed to prepare solutions containing lower concentrations of free iodine for use as antiseptics. Iodine swabs (Iodine Impuls, N.F.) and various types of applicators are marketed; all usually depend upon the free iodine present.

"Heliogen" is a mixture of chloramine-T, potassium iodide, and inert ingredients. Free iodine is released when moisture is present or water is added. Ryan *et al.* (1949) reported favorably on the use of this product in various deep wound infections. Tablets which liberate 25 mg of free iodine are also available and have been recommended for the preparation of a mouth wash for use as an adjunct in home care for the maintenance of periodontally treated patients (Berliner, 1953).



The Compound Dental Liniment of Aconite and Iodine, N.F. VII is an antiseptic. The latter, as in the case of other iodine-aconite, iodine-myrrh, and iodine-nutgall mixtures, though antiseptic, are used primarily as counterirritants to the gingiva. Iodine and Zinc Iodide Glycerite (Diluted Talbot's Solution), N.F. IX, is employed in oral surgery, usually diluted with water before use to suit varying conditions.

Preparations containing free iodine with varying percentages of glycerin (iodoglycerols) or propylene glycol (iodoglycols) or with tannic acid (iotangen), some of which are known as "paints," are employed as antiseptics for infected mucous membranes. "Mandl's Paint," containing iodine 1.25 per cent w/v, with potassium iodide and oil of peppermint in glycerin, is widely used for throat infections.

Iodized Cotton, Iodine Varnish (benzoin combinations) and Collodion Iodine (Chilblain Paint), official in N.F. IV and used on unbroken chilblains, are available.

A combination of iodine and boric acid (Boroiodine) in intimate powder form is used as a dusting powder for wounds and by insufflation especially in the treatment of chronic uncomplicated suppurative otitis media. The fine impalpable powder usually contains 1 to 2 per cent iodine.

"Iodized Phenol," known also as "phenol iodide," should not be mistaken for *p*-iodophenol, which see. The former name is applied to a mixture (liquid) of iodine, 1 part and phenol, 4 parts. Also available is "Phenolated Iodine Solution," N.F. VIII, known also as "Carbolized Iodine Solution" or "French Mixture" or "Boulton's Solution." It is an aqueous solution containing 0.6 per cent liquified phenol, 1.5 per cent Lugol's solution, and 16.5 per cent glycerin, and is used undiluted by topical application as a bactericide and fungicide.

A solution of iodine in liquid petrolatum, with or without menthol or phenol and other therapeutic agents, is used as an antiseptic spray through an atomizer, by drops, or as a nasal pack in upper respiratory infections.

Iodine ointments as antiseptics for the treatment of minor cuts, abrasions and wounds are available. Some are marketed under trade names such as Iodex and Vodine.

"Iodex" contains iodine in organic combination, loosely combined with a fatty acid carrier.

"Vodine," containing 2 per cent iodine and 2.4 per cent sodium iodide in a thermostable water-miscible ointment base, possesses no iodine odor until the ointment is brought in contact with the skin. It is a stable Iodine Solusolve which does not stain, permits

the use of bandages, and may be washed off readily with soap and water (Kass, 1949).

Combinations of iodine and ichthyol (iodoichthyol or ichtholdine) are employed topically as antiseptics.

*Iodophors*.—Surface activating agents serve as carriers and as solubilizing agents for iodine. Anionic, cationic and non-ionic compounds are iodine carriers and differ only in the degree of their reactivity with iodine and the amount of iodine they can carry. The combination of iodine and carrier is known as an "iodophor." The characteristics of iodophors are usually a display of an enhanced bactericidal activity of iodine, a reduction of the vapor pressure, odor and staining almost to the vanishing point, and they can be readily diluted with water.

In a recent presentation (Lazarus, 1952), an iodophor compound gave excellent cleaning and sanitizing results in dairy farm sanitation on utensils, rubbers, milking machines and udders. It also was reported that "against FDA *S. typhosa*, 25 ppm of the iodophor compound sterilized 12 increments as against 200 ppm available chlorine, which sterilized 3 increments. In both cases, sterility in 30 seconds at 20°C was used as the end point."

Satisfactory findings obtained with this compound, a solution of iodine in a specific nonionic of the alkylphenoxy polyglycol ether type, have been reported (Terry and Shelanski, 1952).

PVP or polyvinylpyrrolidone (Plasdone), the blood plasma extender and retardant vehicle for drugs, forms water-soluble or water-dispersible complexes of indefinite composition, permitting the solubilization of substances ordinarily insoluble in water. It appears to hold onto these substances and then releases them slowly over an extended period of time. PVP combined with iodine is said to be non-irritating, can be used on all tissues, and the effectiveness against bacteria and viruses is increased. It is believed that PVP with iodine can be used not only topically but also orally or even intravenously.

## ORGANIC IODINE COMPOUNDS

Iodoform, N.F. IX,  $\text{CHI}_3$ , was introduced as an antiseptic about 1879. It occurs as a fine greenish-yellow powder or lustrous crystals, possessing a peculiar characteristic, penetrating and persistent odor. Excessive heat should be avoided as it is slightly volatile even at room temperature. Iodoform came into extensive use as a dusting powder, especially to promote granulation and diminish infection of open wounds. Applied to mucous membranes, iodoform exercises a marked anesthetic action. It is itself almost free of antibacterial properties even though its iodine content is

high (96 per cent), but when brought into contact with bodily secretions, it gradually liberates free iodine, which is actively antiseptic. It is used as an antiseptic dressing by topical application in suppurating wounds, gauze impregnated with 5 or 10 per cent of iodoform being inserted as packing to prevent premature formation of granulation tissue and to prevent closure of the wound orifice, so that exudate will drain adequately. Oily suspensions have also been employed in the treatment of tuberculous fistulas. "Unguentum Iodoformi" (U.S.P. X) containing iodoform 10 per cent, wool fat 20 per cent, and petrolatum 70 per cent, is used at times.

Iodoform has been and is employed also as iodoform spirit, iodoform injection (intended for injection into the urinary bladder), iodoform pencils, iodoform paste and soap, iodoform tampons and iodoform varnish (Pigmentum Iodoformi Compositum, B.P.C.). It also is used in suppositories, usually for hemorrhoids. At one time, a 5 per cent solution in flexible collodion was official as Collodium Iodoformi (N.F. IV).

The following ointment pastes have had wide use:

BIPP paste consists of bismuth subnitrate, 1 part; iodoform, 2 parts; petrolatum or liquid petrolatum, 1 part. It is a dressing employed for packing wounds, cutaneous ulcers, fistulous tracts and other cavities, where a prolonged antiseptic action with minimal tissue toxicity is desired. See also Pasta Bismuthi et Iodoformi, B.P.C.

ZIPP paste is prepared as is BIPP paste except that zinc oxide replaces the bismuth compound. It is less costly and avoids the possibility of any bismuth absorption.

ZISP paste is used as a dressing in the treatment of wounds in a manner similar to BIPP and ZIPP pastes. It contains zinc peroxide, iodoform, sulfanilamide and petrolatum.

As a pediculocide, N.C.I. (Pediculosis or Louse Powder), a mixture of 96 parts crude naphthalene, 2 parts creosote and 2 parts of iodoform has been and is used in the treatment of pediculosis.

One of the principal obstacles to the use of iodoform is the penetrating and persistent odor. Attempts to disguise this odor have resulted in a number of marketable preparations similar to Iodoformum Aromatisatum (N.F. IV), which is a mixture of iodoform 96 per cent and coumarin 4 per cent. These combinations generally have not proven suitable with the result that other iodine compounds and derivatives have been introduced to replace iodoform. Thymol iodide (which see) is the one most widely used. Others are:

Bismuth Formic Iodide is a mixture of bismuth oxyiodide, formaldehyde-gelatin and thymol iodide. B-F-I is a powder containing bismuth formic iodide, bismuth subgallate, zinc phenolsulfonate,



amol (mono-n-amyl hydroquinone ether), alum (potash) and boric acid with menthol, eucalyptol, thymol and inert diluents. It is an antiseptic, astringent and fungicide and is used topically as a dry powder or by insufflation.

Bismuth Iodosubgallate (Airoform, Airogen, Airol) is a dark gray, odorless powder used in 10 per cent concentrations as an emulsion or ointment.

Bismuth oxyiodopyrogallate (Gaminaform, Pyroform) is a yellowish-red powder and is also employed externally as a dusting powder.

Bismuth Thymol Iodide, used locally as an antiseptic surgical dressing, contains bismuth oxyiodide, 8 per cent; thymol iodide, 4 per cent; and boric acid, 88 per cent.

Ethyl Diiodosalicylate, Europhen, the isobutyl derivative of iodo-orthocresol, Iodamisol (ortho-iodoanisole), Iodoanisole (Iodo-phenyl methyl ether), Iodolin (Quinoline chloromethylate iodochloride) and Iodocresol (Traumatol) are used in place of iodoform.

Iodoformin, methenamine - iodoform,  $\text{CHI}_3 \cdot (\text{CH}_2)_6\text{N}_4$ , is a whitish or cream-colored, fine powder with a slight iodoform odor. It contains 73.75 per cent iodoform (71.32 per cent iodine) and 26.25 per cent methenamine. Its uses are the same as given for iodoform.

Iodo-Gallicin, bismuth oxyiodomethyl gallol, prepared by the action of bismuth oxyiodide on methylgallate, is a dark gray powder and is used as a substitute for iodoform.

Iodol, tetraiodopyrrol,  $\text{C}_4\text{I}_4\text{NH}$ , is prepared by dissolving pyrrol and iodine in alcohol, allowing it to stand 24 hours and precipitating with water. It is a grayish-brown powder and is used to replace iodoform as an antiseptic dressing in wounds. It also has been marketed as Jadol, Iodolen and Iodopyrrole.

Starch Iodide (Iodized Starch), a bluish-black powder containing about 2 per cent iodine, has been used to replace iodoform.

Thymol iodide, N.F. IX, is a mixture of iodine derivatives of thymol, principally dithymol diiodide,  $(\text{C}_6\text{H}_2(\text{CH}_3)(\text{C}_3\text{H}_7)(\text{OI})_{2,3,4})_2$ , and contains when dried not less than 43 per cent iodine. It also is known as Annidalin, Aristol, Iodistol, Iodosol, Iodothymol, Iosol, Lothymol, Thymiodol and Thymodin. It is a reddish-brown or reddish-yellow powder with a very slight aromatic odor. It is insoluble in water or glycerin and only slightly soluble in alcohol and soluble in chloroform, ether, oils and collodion. Thymol iodide is employed as a nontoxic and nonirritant antiseptic chiefly in the form of a dusting powder, pure or with equal parts of boric acid; as a spray or ointment, 5 to 10 per cent in liquid petrolatum or ointment-base; or as a suppository (0.325 to 0.975 g). Due to freedom from odor, it is used to replace but is apparently less efficacious as an antiseptic than iodoform.

## OTHER IODINE COMPOUNDS

The following are other iodine compounds used as antiseptics:

Alkylamine hydriodides, aluminum iodide, mercuric iodide, bismuth iodide, methyl stannic iodide (Staniform), and colloidal silver iodide (Neo-Silvol, Argiod, Alb-Argentum) are iodine derivatives which are used as antiseptics. However the latter activity is frequently due in great measure to the metallic or positive element present.

Calcium iodate (Calcinol), a white, odorless powder, is used in various concentrations in mouth washes, gargles and as a dusting powder as a deodorant and antiputrefactive.

Diiodophenolsulfonic Acid (Soziiodolic Acid) is used topically in 2.5 per cent aqueous concentrations in the treatment of pharyngitis. Dijozol is a solution of a salt of this acid used locally as an antiseptic.

Iodaminal is a combination of iodine and amino acids, used as an antiseptic.

Iodic Acid,  $\text{HIO}_3$ , generally used in the form of its salts, usually sodium iodate, has been used as an antiseptic, astringent, caustic and deodorant in concentrations from 0.2 per cent to 2 or 3 per cent as a gargle, wound irrigation, or for throat swabbing. Sodium iodate also is used in dilute solutions or diluted with boric acid as a dusting powder for cutaneous ulcers, septic wounds, affections of mucous membranes and in otorrhea.

Iodine Tribromide in a concentration of 1:300 aqueous solution has been used as a spray in various mouth and throat affections.

Iodine Trichloride ( $\text{ICl}_3$ ) in concentrations of 0.1 per cent to 1 per cent aqueous solution is used as an antiseptic. "A 0.1 per cent solution kills the vegetative forms of bacteria in 1 minute and a 1 per cent solution destroys spores within 5 to 10 minutes" (Zinsser and Bayne-Jones, 1939). "Atomide" is a brand of iodine trichloride in 1 per cent aqueous solution employed as an external antiseptic.

Iodine Monochloride is also used in such concentrations as the trichloride.

Iodocholeate is prepared by treating natural animal bile products, dried oxgall extract, or tauro- and glyco-cholic acids extracted therefrom, with iodine. Alcoholic solution or an ointment containing this substance is used for the treatment of fungus skin infections.

*p*-Iodophenol, containing iodine 57.7 per cent and phenol 42.7 per cent, is obtained by the action of iodine in alkaline potassium iodide upon phenol or by the diazotization of *p*-aminophenol and

subsequent replacement of the diazonium group by iodine. It has been used in various concentrations, usually in glycerin, for skin, throat and gynecological conditions. This compound must not be confused with Iodized Phenol.

U-F-I, Urea Formic Iodide, is a mixture of methylene diureide, dimethylene diureide, free urea and ionizable iodine. Supplied as a powder, ointment, tablet or solution, it is used topically for the treatment of infected wounds and as an antiseptic for irrigating cavities and sinuses.

## IODONIUM COMPOUNDS

These compounds have the general formula  $R_2IX$  (where R is an organic radical and X an inorganic or organic anion) and are true salts, the group  $R_2I$  being a monovalent cation similar to the quaternary ammonium radicals. The iodine occurs in trivalent form. The oxidation state of the iodine in these substances is not clearly understood but the compounds may be regarded as derivatives of hydrogen iodide just as the quaternary ammonium salts are derivatives of ammonia.

The iodine in the iodonium compounds is present as an integral part of their structure as positive ions. They are strong bases and form stable salts when neutralized with acids. In this respect they behave as do the quaternary ammonium compounds and tertiary sulfonium compounds. They resemble the "onium" derivatives in that they are decomposed by heat. The temperature at which decomposition occurs varies with the electronegativity of the negative ion.

Antibacterial efficiency tests of several iodonium compounds were conducted in the author's laboratory (Gershenfeld and Witlin, 1948b) with diphenyliodonium chloride, bis-*p*-chlorophenyliodonium sulfate, bis-*p*-bromophenyliodonium iodide, bis-*p*-chlorophenyliodonium iodide, bis-*p*-iodophenyliodonium iodide and diphenyliodonium iodide. The following observations were noted:

"1. These iodonium compounds (in powder form) displayed bacteriostatic activity when tested by the F.D.A. agar plate technique (inhibition zones from 11 to 15 mm).

"2. Only bis-*p*-chlorophenyliodonium sulfate in saturated aqueous solution (even when diluted 1:4) displayed bactericidal efficiency against *M. pyogenes* var. *aureus* at 37°C within 1 minute.

"3. The addition of sodium thiosulfate did not affect the bacteriostatic or bactericidal efficiencies of the iodonium compounds. Solutions did not reveal the presence of free iodine.



"4. Saturated solutions of the iodonium compounds in ethyl alcohol (95 per cent) displayed bactericidal efficiencies greater than alcohol itself, against *M. pyogenes* var. *aureus* at 37°C within 1 minute.

"5. Saturated solutions of all the iodonium compounds in a solvent consisting of 10 per cent acetone by volume in alcohol (95 per cent) displayed bactericidal efficiency against *M. pyogenes* var. *aureus* at 37°C within 1 minute.

"6. A saturated solution of bis-*p*-chlorophenyliodonium sulfate in acetone-alcohol solvent displayed bactericidal efficiency within 1 minute against *S. typhosa*, *Serratia marcescens*, *P. aeruginosa*, *E. coli* and *Proteus vulgaris*.

"7. A saturated alcohol-acetone solution of bis-*p*-chlorophenyliodonium sulfate (even in dilution) was capable of killing *Bacillus subtilis* (24-hour-old culture) and *B. subtilis* spores (4 day-old culture) within 4 hours at 37°C but was devoid of killing effect within 240 hours at 25°C."

In a later study (Gershenfeld and Witlin, 1948c), the following findings were noted when:

"1. Bacteriostatic efficiency tests were performed on the following iodonium compounds in aqueous solution: Diphenyliodonium chloride, bis-*p*-chlorophenyliodonium sulfate, bis-*p*-bromophenyliodonium iodide, bis-*p*-chlorophenyliodonium iodide and diphenyliodonium iodide. The bacteriostatic efficiencies of these iodonium compounds were tested against *M. pyogenes* var. *aureus*, *Serratia marcescens*, *S. typhosa*, *P. aeruginosa*, *E. coli*, *P. vulgaris*, *B. subtilis*, *B. mesentericus*, *B. megatherium* and *Streptococcus hemolyticus*.

"2. The minimum bacteriostatic concentration of the iodonium compounds varied from 0.001 mg/cc for bis-*p*-chlorophenyliodonium iodide (*M. pyogenes* var. *aureus*) to 0.8 mg/cc for bis-*p*-chlorophenyliodonium sulfate (*P. vulgaris*). The bacteriostatic efficiency against some of the test organisms was greatest for bis-*p*-chlorophenyliodonium iodide, followed by bis-*p*-iodophenyliodonium iodide.

"It is of interest to note that three of these iodonium compounds—bis-*p*-chlorophenyliodonium iodide, bis-*p*-iodophenyliodonium iodide and diphenyliodonium iodide—displayed bacteriostatic properties only against the gram positive test bacteria employed, including spore formers, but possessed no effect against the gram negative organisms used in this study. Diphenyliodonium chloride was effective against all of the test bacteria (gram positive and gram negative, including spore formers). Bis-*p*-chlorophenyliodonium sulfate had no effect against *S. typhosa*; bis-*p*-bromophenyliodonium iodide had no effect against *S. typhosa* and *E. coli*; but

both compounds displayed bacteriostatic properties against the other test organisms.

"3. In the case of the diphenyliodonium salts, the chloride has a much greater bacteriostatic efficiency than the iodide in all except possibly two cases, against *M. pyogenes* var. *aureus* and *S. typhosa*. With the bis-*p*-chlorophenyliodonium salts, the iodide is more effective than the sulfate in 3 of the 8 cases in which a comparison is possible and about equally effective in the others. It is also to be noted that, in general, bis-*p*-chlorophenyliodonium iodide is about as effective as diphenyliodonium chloride. These observations suggest that bis-*p*-chlorophenyliodonium chloride should have better bacteriostatic properties than any of the compounds mentioned.

"4. In the over-all picture, considering all test bacteria, diphenyliodonium chloride appears as the most generally effective substance. It was bacteriostatic to all organisms tested, only one, *S. typhosa*, requiring a concentration higher than the saturation concentration of the compound of minimum solubility, bis-*p*-bromophenyliodonium iodide. Furthermore, it showed bacteriostatic efficiency at lower concentrations than any of the other compounds tested in the case of 6 of the 11 organisms for which a reasonable comparison is possible.

"Bis-*p*-bromophenyliodonium iodide appears as second in general effectiveness. It was ineffective against two organisms; in 1 case, *S. typhosa*, this can be attributed to its low solubility, while there appears to be a possibility that it would have been effective against *E. coli* in concentrations approaching saturation. Its effective concentration was lower than that of all other compounds in only 2 cases, but it was generally effective at the relatively low concentration of 0.009 mg/cc.

"Bis-*p*-chlorophenyliodonium sulfate was bacteriostatic to one more organism than bis-*p*-bromophenyliodonium iodide but it required concentrations much higher than the saturation value of the other in 4 cases and almost as high in 2 cases.

"Bis-*p*-chlorophenyliodonium iodide was most effective in only 2 cases, required 100 per cent greater concentration than diphenyliodonium chloride and a 50 per cent greater concentration than bis-*p*-bromophenyliodonium iodide in 5 cases. It was ineffective in 5 cases even though its solubility is 50 per cent greater than that of the bromo compound.

"Bis-*p*-iodophenyliodonium iodide required higher concentrations than all other compounds except diphenyliodonium iodide to be effective against two, third most effective against one and was completely ineffective against five organisms even though its solubility is twice that of the bromo compound.

"5. It is of interest to note that when comparing the results of diphenyliodonium chloride and iodide with those of bis-*p*-chlorophenyliodonium sulfate and iodide, it appears that it is the anion which has the effect on the activity.

"6. White mice (Wistar strain) were given intraperitoneal injections of diphenyliodonium chloride and bis-*p*-chlorophenyliodonium sulfate. Animals receiving injections of these iodonium compounds revealed increased excitability, increased respiration and paralysis of the hind legs. The lethal dose was 20 mg/kilogram of body weight for both of the compounds tested."

Diphenyliodonium chloride, also marketed as a dilute aqueous solution, "Katiodin," is employed as an antiseptic and also used for the disinfection of instruments.

*Fungistatic Activity.*—In the author's laboratory (Gershenfeld and Kravitz, *unpublished data*), 17 iodonium compounds, as powders and in saturated aqueous solutions, were tested individually against 11 different fungi.

The 11 test organisms employed were: *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton schoenleini*, *Monilia tropicalis*, *Torula histolytica*, *Monilia krusei*, *Trichophyton violaceum*, *Monilia stelloidea*, *Monilia albicans*, *Trichophyton concentricum* and *Trichophyton tonsurans*. The following 17 iodonium compounds were examined: diphenyliodonium chloride, diphenyliodonium iodide, bischlorophenyliodonium chloride, bischlorophenyliodonium iodide, bischlorophenyliodonium sulfate, bisiodophenyliodonium chloride, bisiodophenyliodonium iodide, bisiodophenyliodonium sulfate, diphenyliodonium tri-iodide, bis(*p*-*tert*.-butylphenyl)iodonium triiodide, bischlorophenyliodonium iodide, bis(*t*-amylphenyl)iodonium iodide, bis(*l*-methylcyclopentylphenyl)iodonium iodide, bis(fluorophenyl)iodonium iodide, bis(triphenylstannophenyl)iodonium iodide, bis(acetamidophenyl)iodonium iodide, and bis(acetophenyl)iodonium iodide.

The following compounds in powder form displayed antifungal activity against all of the test organisms, the effectiveness being indicated in the order named: diphenyliodonium chloride, bis(*t*-amylphenyl)iodonium iodide, bis(acetophenyl)iodonium iodide, bisiodophenyliodonium sulfate, diphenyliodonium triiodide, bischlorophenyliodonium iodide, bis(acetamidophenyl)iodonium iodide, bischlorophenyliodonium chloride and bisiodophenyliodonium chloride.

The other iodonium compounds in powder form did not display any antifungal activity or only very slight activity against a few of the test organisms.

Saturated aqueous solutions of the seventeen iodonium compounds were prepared and tested for fungistatic activity. The solu-



ions were stored at room temperature (20° to 22°C) and their antifungal activity noted at the end of 2 months.

Freshly prepared aqueous solutions of the iodonium compounds revealed the following:

*T. mentagrophytes* was inhibited markedly by bisiodophenyl-iodonium sulfate, diphenyliodonium chloride, diphenyliodonium iodide, bischlorophenyliodonium chloride, and only slightly by bis(triphenylstannophenyl)iodonium iodide, bisiodophenyliodonium iodide, bis(fluorophenyl)iodonium iodide, bisiodophenyliodonium chloride, and bischlorophenyliodonium iodide.

Diphenyliodonium iodide inhibited *M. tropicalis*.

Bischlorophenyliodonium iodide inhibited *T. violaceum* and *T. concentricum* while bisiodophenyliodonium chloride and bisiodophenyliodonium iodide displayed but slight zones of inhibition against these organisms.

Bisiodophenyliodonium sulfate showed large zones of inhibition of the *Trichophytons* (*mentagrophytes*, *rubrum*, *violaceum*, and *consurans*), but proved ineffective against the other test organisms.

Bis(fluorophenyl)iodonium iodide and bis(acetamidophenyl)-iodonium iodide inhibited only *T. violaceum*, and bis(triphenylstannophenyl)iodonium iodide showed but slight inhibition of *T. mentagrophytes* and *M. tropicalis*.

Two-month-old saturated aqueous solutions of the iodonium compounds under test revealed that:

Diphenyliodonium chloride, bischlorophenyliodonium chloride, and bisiodophenyliodonium sulfate were effective against *M. albicans* moderately and all of the *Trichophytons* markedly with the exception of *T. concentricum*; whereas previously they had inhibited only *T. mentagrophytes* in the first two instances and all the *Trichophytons* except *T. concentricum* and *T. schoenleini* more effectively in the third case.

Bisiodophenyliodonium chloride showed mild inhibition of all the *Trichophytons* except *T. concentricum*, a slight increase in activity except for *T. concentricum*, while diphenyliodonium triiodide displayed only a very slight inhibition, although somewhat stronger than the fresh material, against *T. mentagrophytes*, *T. rubrum*, and *T. schoenleini*.

The other iodonium compounds, as saturated aqueous solutions, did not display antifungal activity against the test organisms.

## PROTOZOACIDE

Iodine, its derivatives and combinations are being widely used as protozoacidal agents especially in their effectiveness against

ameba and trichomonads. The following preparations have been found useful:

Chiniofon, U.S.P. XIV (Anayodin, Iodomoebin, Quinoxyl, Loretin, Yatren), is a canary-yellow powder, odorless or with not more than a slight odor. It is a mixture of 80 per cent 7-iodo-8-hydroxyquinoline-5-sulfonic acid,  $C_9H_4NI(OH)SO_3H$ , its sodium salt, and 20 per cent sodium bicarbonate, containing not less than 26.5 per cent and not more than 29 per cent iodine. Chiniofon has a bitter taste, but it leaves a distinctly sweetish aftertaste. It effervesces when mixed with water. The sodium salt dissolves in water to the extent of 4 parts per hundred. Chiniofon is effective in all forms of acute and chronic intestinal amebiasis; it is employed in the treatment of carriers, or apparently asymptomatic "cyst-passers," of cases of amebic diarrhea, of cases of acute and chronic amebic dysentery, and of amebic abscess of the liver. It is used orally or by enemata or both. Chiniofon is contraindicated when damage to the liver or thyroid disturbance exists.

Diiodohydroxyquinoline, U.S.P. XIV (5,7-diiodo-8-quinoline, Diodoquin, Abitrene, Direxiode, Embequin, Enterosept, Mixiod, Yodoxin),  $C_9H_4NI_2OH$ , occurs as a colorless or light yellowish to tan microcrystalline powder and contains not less than 60.5 per cent and not more than 64 per cent iodine. It is employed orally as an antiprotozoan agent in the treatment of intestinal amebiasis, including carriers, and also as a trichomonicide in the treatment of *Trichomonas hominis* (intestinalis) infections.

Iodochlorhydroxyquin, U.S.P. XIV (5-chloro-7-iodo-8-quinolinol, Nioform, Vioform),  $C_9H_4NClI(OH)$ , is a grayish-yellow powder with a faint odor, mp  $172^\circ C$  with decomposition. It contains approximately 39.7 per cent iodine and is almost insoluble in water and sparingly soluble in alcohol. It was first introduced as an antiseptic dusting powder but is now chiefly used internally as an amebicide and is also employed topically against *Trichomonas vaginalis* vaginitis. The undiluted powder is dusted on wounds, cutaneous ulcers, burns, and skin eruptions such as atopic dermatitis, eczema of the external auditory canal, eczema of the legs, scalp, scrotum and perineum impetigo, and also in chronic dermatitis, acute psoriasis, and intertriginous psoriasis. It may be used externally as a 2 per cent lotion, paste or ointment. A powder containing 25 per cent of this compound with boric acid, lactic acid, lactose, and zinc stearate (Compound Iodochlorhydroxyquin Powder) is official in the U.S.P. XIV.

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## 10

# MERCURIALS – INORGANIC AND ORGANIC

### INTRODUCTORY HISTORICAL REVIEW

HISTORICALLY, the use of mercury and its compounds is of comparatively recent date. Mercury was unknown to the ancient Jews and its value was not recognized by the Greeks and Romans. About 300 B.C. its preparation was described by Theophrastus and it became a substance of great importance to the early alchemists. Its first use for skin affections was by the Arabian physicians and it was used in the form of vapors or in a finely divided state. In the 16th century, when chemistry and scientific medicine were beginning to merge, mercury played a very important role in the various theories and its therapeutic value in the treatment of intestinal disorders and skin diseases was well recognized. Mercury and its salts in certain forms are non-toxic and as much as 1 pound of mercury has been given for intestinal obstruction without ill effects. It may also be said, in general, that the mercurous salts are relatively non-toxic. However, certain of the mercuric salts may be very toxic and it is this variation in toxicity that has accounted for the fluctuating value of these compounds in medicine.

It will be seen from an historical review that before the germ theory of disease was well established, it was a well-recognized fact that the use of mercurial salts would prevent sepsis. Since the time of the early Arabian physicians, substances were clinically evaluated on their ability to aid in healing and the reports in the

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literature describe the prevention of sepsis in open wounds by the use of mercurials with hundreds of years of untreated infected cases acting as controls. Here is, indeed, the earliest ideal antiseptic test—the ability of these compounds to act as antiseptics under actual conditions of use (Brewer, 1950).

After the germ theory of disease was fully established, Koch (1881) popularized the use of mercury bichloride with his famous string experiment using anthrax spores. Immediately the medical world believed it had in its hands an all-powerful germicide which would destroy any disease producing organism. Koch had apparently shown that very great dilutions of mercury bichloride would kill the spores of the most resistant organisms. It was therefore suggested that this mercurial could be used for instrument sterilization and as a germicide in various medical practices.

Geppert (1889), with carefully controlled experiments, showed that the inorganic mercurial salts were very poor germicides, although they possess an almost unbelievable bacteriostatic property. He used ammonium sulfide to neutralize the mercurial and this was the first of a long series of papers to try to establish the true value of these compounds. These studies have definitely shown that the antibacterial activity of the mercurials is due to their bacteriostatic properties and that they are only slowly bacteriocidal to vegetative cells. If one considers spore-forming organisms, the literature is quite filled with data to show the limitations of the mercurials to act as germicides, although there are quite a number of publications to show that in spite of this limitation they can be relied on as antiseptics where these organisms are concerned. Seymour-Jones (1911) published an anthrax sterilization method which made use of corrosive sublimate. Proof that mercury bichloride was not satisfactory for killing spores has been demonstrated by Geppert (1889), Heider (1892), Xylander (1907), Croner and Naumann (1911).

More recently, Moll (1920) demonstrated, with the aid of animals and cultural experiments, that mercury bichloride could not be depended on to kill tetanus spores. Tizzoni and Cattani (1891) had found mercury bichloride unsatisfactory for killing *Clostridium tetani*, but one cannot make sure that spores were present in their experiments.

Reports on the ability of the other non-proprietary mercurials vary from favorable, when non-sporulating organisms were used in attempts to show that they are no more active than mercury bichloride on spores. Mackie (1928) and Bulloch (1929) have shown that mercuric iodide, potassium mercuric iodide and mercuric cyanide were unsatisfactory for sterilizing spores on catgut.

Raiziss and Severac (1923) report a series of experiments with Metaphen. These experiments indicate that Metaphen is eleven times more powerful than mercury bichloride when tested against the spores of *Bacillus anthracis*, with an exposure of one hour.

Powell and Jamieson (1931) state, "The active material in Merthiolate is 40 to 50 times as effective as phenol upon *S. aureus* and *B. typhosus*, *B. coli*, various streptococci, and the tetanus bacillus were all found to be in this general range of susceptibility to Merthiolate."

Birkhaug (1933) reports the highest dilution of disinfectant killing *Bacillus subtilis* after 10 minutes, but not after 5 minutes, contact at 37°C as follows: Phenyl mercuric nitrate 1:65,000, Merthiolate 1:24,000, Metaphen 1:36,000, Mercurochrome 1:300, mercury bichloride 1:120,000. Simmons (1928) reported that Mercurochrome would not kill the spores of *B. anthracis* in 10 hours. Scott, Hill and Ellis (1929), however, found *B. anthracis* to be killed in 10 minutes. Weed and Ecker (1932) report that phenyl mercury nitrate kills *B. subtilis*, *Cl. septicum*, *Cl. tetani*, *Cl. welchii*, *Cl. histolyticum*, *Cl. sporogenes* and *Cl. chauvei* in 10 minutes or less. Clock (1933) reports that he was unable to sterilize catgut, artificially infected with a sporulating organism, using potassium mercuric iodide, Metaphen, Mercurochrome, Merthiolate or Mercurophen. McCleskey and Swingle (1937) found in using Mercresin and Mercarbolid that spores of *Cl. welchii* were not killed after exposure of 120 hours. Scott (1937) gives the following results: Metaphen was incapable of sterilizing spores of *Cl. chauvei*, *Cl. septicum*, *Cl. novyi* or *B. subtilis*. Merthiolate failed to kill *Cl. chauvei*, *Cl. septicum* or *Cl. novyi* in less than 3 days. Phenyl mercury nitrate failed to sterilize *Cl. septicum* and *Cl. tertium* in less than 3 days. Ecker and Smith (1937) suggest the use of merphenyl nitrate for sterilizing several pieces of surgical equipment. They state that potassium mercuric iodide failed to kill certain organisms in their experimental work. Although this review of the literature bearing on the ability of the mercurials to kill spores lays no claim to completeness, it is at least representative of the work done in which spores were used.

Most of the results in which strong bactericidal action against spore-forming organisms is reported were obtained by phenol coefficient or similar methods which do not take inhibition into consideration. In those instances in which recovery of the organism is reported, the technique used does not make sure that all organisms were exposed to the disinfectant for the full period of testing.

In reviewing the previous work on the mercurials, one is impressed that with the advent of the sulfonamides and later the



antibiotics, the mercurials have not received the attention in medical literature that they did for some 60 years. The mercurials were never satisfactory systemic chemotherapeutic agents, although when the less toxic organic mercurials were first introduced they were suggested for chemotherapy and voluminous literature exists concerning their use in this field. Now with satisfactory agents for chemotherapy, the mercurials have assumed their proper place in medicine as local antiseptics and preservatives in biologicals and industry. It will then be the purpose of this chapter to present data so that one may truly establish the value and limitations of these compounds.

## TYPES OF MERCURIAL COMPOUNDS

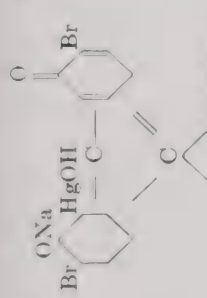
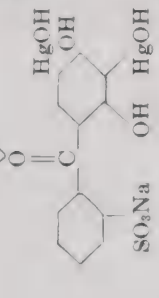
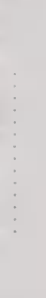

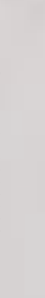

It was pointed out earlier that mercury was first used in medicine for skin disorders as an inunction in a finely divided state. The value acclaimed for this treatment was probably due not to the mercury but to some of its simpler salts; for in this finely divided state it was readily converted into any one of a number of compounds.

Mercury bichloride, the first salt popularized as an antiseptic, possessed several disadvantages which were known and described by Koch (1881): It corrodes metal instruments, its "killing" effect is diminished by organic matter and serum, and it coagulates protein. It cannot be used on the skin over long periods of time without causing dermatitis and is toxic when injected parenterally. Because of these limitations, other simple salts of mercury were soon tried and several of them soon became popular since they did not precipitate albumen. None of the inorganic compounds satisfied all of the shortcomings of bichloride and with the additional criticism of Geppert and others that these compounds did not kill spores, chemists throughout the world attempted to produce a mercurial which would not have these disadvantages and perhaps might kill the sporulating organisms as well. The first products of these researches possessed few advantages over mercury bichloride, but just at the close of World War I several compounds appeared on the open market in the form of organic mercurials. With some types of laboratory tests they seemed to be more effective than the simpler mercurials and they do obviate the corrosive action. The general ability to kill spores, however, has not been substantiated although they may be able to act as antiseptics against these organisms (Brewer, 1950).

McCulloch (1945) in discussing the chemistry of these complex compounds of mercury suggests that, as a rule, the introduction of

TABLE 2. — THE MERCURIALS MORE COMMONLY USED AS ANTISEPTICS (BREWER 1939)\*\*

Name	Manufacturer	Chemical Name	Mercury Content per cent	Structural Formula*	Empirical Formula	Type	Comment	pH	Concentration of Type Used
Merthiolate	Eli Lilly & Co.	Sodium salt of ethyl mercuri thiosalicylic acid	49		$C_2H_5HgS$ $C_6H_4O_2S$ Hg Na	R Hg S R <sub>2</sub>	Where R <sub>1</sub> and R <sub>2</sub> are different organic radicals	9.8	1:1,000 isotonic solution
Metaphen	Abbott Laboratories	Anhydride of 4-nitro-5-hydroxy mercuri ortho cresol	56-57		$C_7H_5O_4Hg$ N	R Hg X		10.5	1:2,500 aqueous germicidal solution
Merphenyl nitrate	The Hamilton Laboratories	Phenyl mercuric nitrate (basic)	63		$C_6H_5HgOH$ , $C_6H_5HgNO_2$	R Hg N	Where R is an organic and X is an inorganic radical	4.2	1:1,500 boric acid solution
Mercurbolide	The Upjohn Co.	Ortho hydroxy phenyl mercuric chloride	60-95		$C_6H_5(OHgCl)$	R Hg N		6.5	1:1,000 aqueous solution
Mereresin	The Upjohn Co.	bichloride Not a chemical entity		A mixture of mercuribolide and five isomeric anyl ortho cresols					
Mertoxol	McKesson & Robbins, Inc.	Acetoxy mercuri-2-ethylhexyl-phenol sulfonic acid	40		$C_{16}H_{21}O_6S$ Hg	R Hg O R <sub>2</sub>		5.4	Tincture (only form available)
								3.5	1:1,000 isotonic solution

Mercurio- chrome	Hynson, Westcott & Dunning	Sodium salt of di-brom hydroxy- mercuri fluo- rescein	24 26		$C_{10}H_4O_3Br_2HgNa_2$	$R_1HgX$	9 1	1:50 aqueous solution
Meroxyl	Hynson, Westcott & Dunning	Sodium salt of 2-4 di hydroxy- 3-5 di hydroxy- mercuri benzo- phenone 2'sul- fonic acid	26 29		$C_{10}H_4O_3Hg_2SNa_4$	$R_1HgX$ $R_2HgX$	7 1	1:200 stabilized aqueous
Mercury oxycyanide	Nonpropri- etary		85 5		$Hg(CN)_2$	Inorganic	9 1	1:6,000 aqueous
Mercuric cyanide	Nonpropri- etary		83 3		$Hg(CN)_2$	Inorganic	6 2	1:1,000 aqueous
Potassium mercuric iodide	Nonpropri- etary		25 5		$K_2HgI_4$	Inorganic	9 2	1:1,000 aqueous
Mercury chloride	Nonpropri- etary	Mercuric chloride, "corrosive sublimite"	83 3		$HgCl_2$	Inorganic	5 9	1:1,000 aqueous

\* The positions of some of the groups are doubtful.

\*\* Some of these mercurials are no longer of commercial importance but are included since they represent certain types of the mercurials.



acid-containing groups in the compound increases the solubility of the compound but lowers its germicidal power. He further states, "The more complex combinations of dyes and mercury frequently exhibit a reversal of the specificity shown by mercuric chloride. This would indicate a different type of reaction rather than merely an increase in the intensity of the reaction. Against mercuric chloride, *M. pyogenes* var. *aureus* is almost twice as resistant as *E. coli* while phenyl mercuric nitrate, Merthiolate and Metaphen, *E. coli* is between 30 and 50 times as resistant as *M. pyogenes* var. *aureus*."

Table 2 shows the mercurials more commonly used. The type of compound may be seen as well as the structural and empirical formula. These compounds range from 24 to 85.5 per cent mercury. In addition to the mercurials shown in this table, Merbak (Schieffelin), 2-acetoxymercuri-4-diisobutyl phenol, which is used in 0.1 per cent concentration, and Phenyl mercuric borate (non-proprietary), which is also used in 0.1 per cent concentration, should be mentioned.

## MECHANISM OF ACTION OF THE MERCURIALS

One of the earliest ideas concerning the mechanism of action of the mercurials is that they precipitate the bacterial protein by combining directly with it to form a mercury-proteinate. This theory came about logically, since mercury bichloride was known to combine directly with the tissues and to coagulate their proteins. Smith (1869) writing on the disinfecting action of metallic salts, states that they prevent sepsis by combining directly with flesh, making a permanent compound and keeping out the contaminating air. As stated earlier, Geppert (1889) had suggested that the sublimate made a chemical bond with the cell, which could be broken by converting the mercury to the sulfide. Krönig and Paul (1897) pointed out that the bactericidal action was due to the free ions of mercury in the solution and not to the molecular concentration. Various authors have enlarged on this protein precipitation theory, but no additional proof of value has been advanced.

Süpfle and Müller (1920), and Engelhardt (1922) have attempted to show that sublimate is adsorbed on the cortical layer of the bacteria and that, by treating with a stronger absorbent (blood charcoal), the mercurial could be separated from the bacteria so completely that germs apparently dead would renew their growth.

This surface adsorption theory fitted so well into the later observations of colloidal chemists that Bancroft and Richter (1931) attempted to explain chemical disinfection as a simple adsorption of a germicide giving a reversible reaction. The analogy seemed

probable but it was found that it did not compare to the common reversible chemical reactions since the addition of a large number of bacteria slowed rather than accelerated the velocity of the antibacterial action.

A theory which fits in well with our modern conception of cell physiology was first described by Duclaux (1900) in his "Traité de microbiologie." He assumed that chemical disinfection was simply a destruction of the enzymes of the bacterial cells and without these enzymes the bacteria could not live. In light of our present knowledge of enzyme chemistry and bacterial physiology, we know that the enzymes may be reversibly precipitated by heavy metal salts. Thus, we have an excellent explanation of the fact that organisms apparently killed by a mercurial can be revived with suitable neutralizing agents. This theory, further supported by the works of Isaacs (1932), has been tabulated by Gay (1935). The points pertinent to this present work are that in the case of spores, each cell is provided with its maximum enzyme content at the start. According to this theory, the coating of mercury apparently has no effect on the enzyme contained within the spore; thus, theoretically, the spore could exist indefinitely without being affected.

This would serve to explain the survival of the spores in catgut sutures which had been suspended in 3 per cent potassium mercuric iodide for more than 10 years (Brewer, 1937).

This theory would also explain the necessity for having a medium containing the essential elements for the optimum growth of the organisms, for it has been found that injured cells may grow on one medium and not on another.

It has been demonstrated that all culture media contain a certain varying amount of mercurial neutralizer and that by appropriate treatment lead subacetate would precipitate  $S^-$  ions. The bacteriostatic activity of the mercurial was enhanced in a comparative media titration, whereas that of cationic and anionic detergents was unaffected. The addition of sodium thioglycollate as  $SH^-$  would reverse this action (McLaughlin and Brewer, 1951).

Another theory concerning the mechanism of bactericidal action, although not applicable to all the mercurials, is at least worthy of mention. This theory was suggested by Stark and Montgomery (1935) to explain the bactericidal action of merphenyl nitrate (first described by Otto in 1870 as phenyl mercuric nitrate). Later examinations indicate that the product is phenyl mercuric hydroxyl nitrate (basic nitrate). It is suggested that merphenyl nitrate differs from other complex mercury compounds in that the mercury-containing ion is the cation ( $C_6H_5Hg$ ). Since bacteria usually carry a negative charge, the high bacteriostatic power of

this compound is attributed to the positive charge of the mercury-containing ion.

Fildes (1940), in his very excellent review, suggests that the mercurials possibly act as inhibiting agents by interfering with an essential metabolite. Since the antibacterial action may be prevented or antagonized by hydrogen sulfide, it is assumed that the

TABLE 3.—ANTI-MERCURIAL EFFECT OF SULPHUR COMPOUNDS\*

$\text{HgCl}_2$ ,  $8 \times 10^{-6} M$ , was autoclaved in water and 1 ml added per tube. Thiolacetic acid,  $10^{-1} M$  in  $N/1$   $\text{HCl}$ , was autoclaved, neutralized and diluted  $10^{-2} M$ . Glutathione and cysteine  $\text{HCl}$  were filtered as  $10^{-2} M$  solutions. Cystine was  $5 \times 10^{-2} M$  (as S) in  $N/10$   $\text{HCl}$ , filtered and diluted to  $10^{-2} M$ . Methionine,  $10^{-2} M$ , was filtered. The inoculum was added last.

Flask		<i>Hg</i> ( $8 \times 10^{-6} M$ )	<i>S.</i> compounds ( $\times 10^{-6} M$ )	Growth in hours and days		
				21	44	3
1	Medium	0		tr.	++	+++
2	"	+		0	0	0
3	"	+	thiolacetate	tr.	++	+++
4	"	+		?	+	+++
5	"	+		0	0	0
6	"	+	cysteine	tr.	++	+++
7	"	+		tr.	+	++
8	"	+		0	0	0
9	"	+	glutathione	tr.	++	+++
10	"	+		tr.	++	+++
11	"	+		?	+	++
12	"	+	cystine	0	0	0
13	"	+		0	0	0
14	"	+		0	0	0
15	"	+	methionine	0	0	0
16	"	+		0	0	0
17	"	+		0	0	0

Plus signs are roughly proportional to the mass of growth.

tr. = trace.

? = doubtful.

\* From paper, "The Mechanism of the Anti-Bacterial Action of Mercury," by Paul Fildes, which appeared in the April, 1940, Vol. XXI, No. 2, edition of The British Journal of Experimental Pathology. From the Department of Bacterial Chemistry (Medical Research Council), Bland Sutton Institute of Pathology, and from the Courtland Institute of Biochemistry, Middlesex Hospital, London, W. 1. (Received for publication February 14, 1940.) Pages 67-73.

insoluble sulfide is formed, thereby removing the active mercury ions. It is well known that  $R-SH$  compounds are essential metabolites in all cellular physiology and he definitely suggests that the antibacterial action of mercury is due to a similar interference with this essential  $R-SH$ .

On a molecular basis, Fildes has shown that the antagonistic action of glutathione is greater than that of thiolacetic acid. If this antagonistic action were due solely to  $SH$ —the two should be equal since their combining portions are the same.



Even though there are discrepancies in Fildes' data, it may be that his conclusion, "Within the limits of low Hg concentrations and low temperature, the antibacterial action of mercury is reversible by  $-SH$  and it is concluded that the action of mercury is merely to inactivate the  $-SH$  group without demonstrable injury to the cell," may be justified.

TABLE 4.—QUANTITATIVE RELATIONSHIP BETWEEN GLUTATHIONE AND Hg IN GROWTH\*  
(For details see previous table)

Flask				Growth in hours and days			
		$-SH$ ( $\times 10^{-5} M$ )	$Hg$ ( $\times 10^{-5} M$ )	24	43	3	4
1	Medium	+	0	+	+++	+++	+++
2	"	+	20	0	0	0	0
3	"	+	20	0	0	0	0
4	"	+	20	0	+	++	+++
5	"	+	20	tr.	++	+++	+++
6	"	+	10	0	0	0	0
7	"	+	10	0	0	0	0
8	"	+	10	0	++	+++	+++

TABLE 5.—QUANTITATIVE RELATIONSHIP BETWEEN THIOLACETATE AND Hg IN GROWTH\*

Flask				Ratio:		Growth in 2 days
		$-SH$ ( $\times 10^{-6} M$ )	$Hg$ ( $\times 10^{-6} M$ )	$Hg/-SH$		
1	Medium	+	0	0	..	+++
2	"	+	800	64	1/12 5	0
3	"	+	"	32	1/25	+++
4	"	+	"	16	1/50	+++
5	"	+	"	8	1/100	+++
6	"	+	400	32	1/12 5	0
7	"	+	"	16	1/25	+++
8	"	+	"	8	1/50	+++
9	"	+	"	4	1/100	+++
10	"	+	200	32	1/6 25	0
11	"	+	"	16	1/12 5	0
12	"	+	"	8	1/25	+++
13	"	+	"	4	1/50	+++
14	"	+	100	16	1/6 25	0
15	"	+	"	8	1/12 5	0
16	"	+	"	4	1/25	0

\* From paper, "The Mechanism of the Anti-Bacterial Action of Mercury," by Paul Fildes, which appeared in The British Journal of Experimental Pathology, April, 1940, Vol. XXI, No. 2, pages 67-73. From the Department of Bacterial Chemistry (Medical Research Council), Bland Sutton Institute of Pathology, and from the Courtland Institute of Biochemistry, Middlesex Hospital, London W. 1. (Received for publication February 14, 1940.)

Selzer and Baumberger (1942) found that mercury has an inhibitory effect on the respiration of yeast cells and the point of attack lies probably in the enzyme system. They assume the inhibition of the respiration by the finely divided mercury is caused by an oxidation—complex formation between the  $SH$  groups of the enzymatic systems of the yeast cells and the mercury.

This work would lead one to believe that more than one enzyme is affected since the consumption of oxygen by yeast is inhibited when the substrate is glucose but not inhibited when lactic or pyruvic acids are used.

Barron and Singer (1945), in discussing the inactivation and reactivation of —SH enzymes, conclude that succinoxidase is inhibited by a mercurial and can be completely reactivated by glutathione when the latter was added 10 to 15 minutes after the mercurial; only 85 per cent reactivation at 30 minutes, and less than 70 per cent reactivated at 1 hour.

TABLE 6.—RESULTS OF INTRAPERITONEAL INJECTIONS INTO MICE OF VARIOUS DILUTIONS OF STREPTOCOCCUS C 203 CULTURE WHICH HAS BEEN EXPOSED TO "MERCUROCHROME" FOR 10 MINUTES AND THEN INJECTED WITH BAL\*

Dilution of C 203M	Ten mice received each dilution Number of Mice Dead at End of 7 Days			
	Infusion Culture Control	C 203M 1% "Mercurochrome" Mixture		
		No BAL	BAL—2 Hrs.	BAL—6 Hrs.
1:10	10	2	10	1
1:1,000	10	0	10	0

Numbers represent the deaths among 10 mice receiving injections in each dilution.

BAL control—10 mice had intramuscular injections of 0.1 ml 1 per cent BAL—no deaths resulted.

A virulent culture of C 203M was diluted 1:10 and 1:1,000 in 1 per cent "Mercurochrome." After 10 minutes exposure of the organisms to the mercurial compound, 50 mice received intraperitoneal injections of 0.1 ml of the 1:10 dilution and 50 mice the 1:1,000 dilution.

At intervals of 2 and 6 hours, 10 mice with injections of each dilution received 0.1 ml 1 per cent BAL in oil intramuscularly.

\* Brewer: 1948, "Reduction of Infectivity of Certain Pathogenic Bacteria by "Mercurochrome." J. A. M. A., 137, 858-861.

This time factor is evidenced in an *in vivo* experiment which was designed to show that organisms, although held bacteriostatically, can be reactivated within the animal body. To prove that the mercurial compound affords protection by impairing the infectivity of the organisms, mice were infected with organisms treated with a mercurial. Some of the mice were then given injections of BAL to neutralize the effect of the mercurial and subsequently the animals died; hemolytic streptococci were isolated from their heart blood. On the other hand, the infected-treated mice which did not receive BAL to neutralize the mercurial survived the infection due to the treatment. This indicates clearly that 2, 3-dimercaptopropanol (BAL) neutralized the mercurial agent which was in or on the treated bacteria, and those bacteria freed of bacteriostatic restraint were thereby enabled to cause death. From this experiment it can be said that by holding an organism bacteriostatically, a general infection may be prevented.

If one waits 6 hours after the treated organisms are injected, this reactivation is very much lessened, which would tend to agree with the suggestions of Barron and Singer. This inactivation and reactivation by mercurials is not limited to bacteria alone. Krueger

and Baldwin (1934) have shown that bacteriophage of *M. pyogenes* var. *aureus* can be inactivated completely by mercuric chloride and reactivated by hydrogen sulfide. Klein, Brewer, Perez and Day (1948) have confirmed this work using *M. pyogenes* var. *albus* bacteriophage. With a titer of  $8.7 \times 10^5$  particles per ml and a 1:10,000 dilution of  $\text{HgCl}_2$  with 30 minutes exposure, there was a 99 per cent reduction in titer. In a 30 minute reactivating procedure using 1:50 sodium thioglycollate, there was a marked reversal of the action of the mercurial. These same workers have demonstrated inactivation of viruses by the mercurials confirming the work of Knight and Stanley (1944) and have further shown reactivation with sodium thioglycollate and BAL. (See Table 7.)

TABLE 7. REACTIVATION OF INFLUENZA A VIRUS WITH BAL\*

Virus Control	Inactivation: Virus plus 1:20,000 $\text{HgCl}_2$ (30 minute contact)	Toxicity Control: 1:20,000 $\text{HgCl}_2$ plus 1:300 BAL	Reactivation: Virus plus 1:20,000 $\text{HgCl}_2$ , 30 minute contact. Followed by 1:300 BAL (30 minute contact)
D5, D5, D6, D6, D6, D6, D7, D7, D7, D8, D8, D9, D9, D9	15 mice all living 10th day. Lungs normal	15 mice all living 10th day. Lungs normal	D9, D9, D9, D10, 4+, 4+, 4+, 4+ 4+, 3+, 3+, 2+, 2+, 2+, 1+

Presence of influenza A virus confirmed by reinoculating infected lungs into mice and neutralizing infective agent with PR8 rabbit antiserum. A ratio of 1:20,000  $\text{HgCl}_2$  to 1:300 BAL is equivalent to a molar ratio of 1:256.

TABLE 8.—REACTIVATION OF INFLUENZA A VIRUS WITH SODIUM THIOLYCOLLATE\*

Virus control	Inactivation: Virus plus 1:20,000 $\text{HgCl}_2$ (30 minute contact)	Toxicity control: 1:20,000 $\text{HgCl}_2$ plus 1:50 Sodium Thioglycollate	Reactivation: Virus plus 1:20,000 $\text{HgCl}_2$ , 30 minute contact. Followed by 1:50 Sodium Thioglycollate (30 minute contact)
D7, D7, D8, D9, D10, D10, 3+, 3+, 1+	10 mice all living 10th day. Lungs normal	10 mice all living 10th day. Lungs normal	D10, 3+, 3+, 3+, 2+, 2+, 1+, 0, 0, 0

\* Morton Klein, J. H. Brewer, J. E. Perez and Beatrice Day, 1948. The Inactivation of Influenza Virus by Mercurials and the Reactivation by Sodium Thioglycollate BAL. J. of imm.—59, #2.

In 7 assays using sodium thioglycollate as the reversing agent marked reactivation of the virus was obtained in 6 assays. No evidence of reversal was obtained in one assay. The results of a representative experiment in which reversal was obtained are shown in Table 8.

In addition to the thio compounds, other types of substances have been found to affect the germicidal efficiency of the mercurials.



Since the neutral salts of heavy metals react with amino groups to form double salts of definite composition, one would expect that the dibasic acids (arginine, lysine) should produce twice as much inactivation of mercuric chloride as the mono-basic acids. It has been found, however, that this is not the case, for glycine, aspartic acid, glutamic acid, arginine and lysine all gave the same degree of inactivation. Cystein, which contains a sulfhydryl group, gives more inactivation of the mercurial, as might be expected. This will be seen in Table 9 (Salle and Ginoza, 1943).

TABLE 9.—EFFECT OF AMINO ACIDS ON THE GERMICIDAL EFFICIENCY OF MERCURIC CHLORIDE AGAINST *STAPHYLOCOCCUS AUREUS*\*

Dilution of <i>HgCl<sub>2</sub></i>	Control	Glycine	Aspartic acid	Glutamic acid	Arginine	Lysine	Cystein
1:250	—	—	—	—	—	—	+
1:500	—	—	—	—	—	—	+
1:1000	—	—	—	—	—	—	+
1:2500	—	—	—	—	—	—	+
1:5000	—	+	+	+	+	+	+
1:7500	—	+	+	+	+	+	+
1:12,000	—	+	+	+	+	+	+
1:25,000	+	+	+	+	+	+	+
1:37,500	+	+	+	+	+	+	+

— = no growth; + = growth after 96 hours at 37° C.

\* Proceedings of the Society for Experimental Biology and Medicine, 54, 85, 1943.

## INORGANIC OR SIMPLE MERCURIAL COMPOUNDS

Simple mercurials such as mercuric chloride, mercury cyanide, and potassium mercuric iodide frequently have been employed as antiseptic agents. Most writers list the objections to these heavy metallic salts as: their toxicity and irritating action upon tissues, a tendency to form precipitates with proteins and other organic nitrogenous compounds, and their corrosive action on metals. As has been stated, the development of many mercurial compounds followed the introduction of acid-containing groups or an organic radical which partially or wholly eliminated one or more of the objectionable features of inorganic mercury compounds. Although the simple mercurials have had a lesser role as antiseptics since the advent of the more complex compounds, their use as industrial preservatives has greatly increased. Thus, a brief summary of properties and the present trends of utilization of this group of compounds are considered in this section.

*Mercuric Chloride.*—Bichloride of mercury ( $\text{HgCl}_2$ ) or corrosive sublimate contains 83.3 per cent mercury and is employed usually in a solution of 1:1000, pH 5.9. It is often formulated as compressed tablets containing equal quantities of citric acid to

facilitate rapid solution. The increased acidity in poorly buffered solutions by citric acid to above that of the iso-electric point of most pathogens; and the theoretical consideration of both positively charged mercury ions and bacteria to repel each other, was noted by McCulloch (1945). As a skin disinfectant, Vaichulis and Arnold (1935) recommended a colored alcoholic solution formulated with 0.75 per cent hydrochloric acid.

With a laboratory procedure employed for study of surface active agents, Smith *et al.* (1950) concluded  $\text{HgCl}_2$  to be effective against the tubercle bacillus at a dilution of 1:1000. Surface tension reducing substances enhanced the bactericidal activity (by phenol coefficient test) of mercuric chloride when the pH was adjusted to 4.0. Fisher (1942). The synergistic activity of mercurials and wetting agents often has been reported. In our experience, a combination of  $\text{HgCl}_2$  and sodium lauryl sulfate, which alone were incapable of killing all organisms, was actually germicidal (Lilley and Brewer, 1949).

Siemens and Schreiber (1946) found 1.0 per cent sublimate ointments irritating and reported the addition of 10 per cent salicylic acid to increase this irritation. No skin irritation was noted with an ointment containing a mixture of salicylated ammonia, oxide of mercury and mercuric salicylate. As a rapid skin disinfectant, Gardner (1948) considered  $\text{HgCl}_2$  as either slow in action or without effect.

As evidenced by reports in the literature, mercuric chloride has been investigated for many diverse usages. A 0.2 per cent solution was recommended as a rotary knife disinfectant for control of ring rot of potatoes (Kreutzer *et al.*, 1946). Similarly, 0.2 per cent  $\text{HgCl}_2$  solution containing 1.0 per cent acetic acid, or 0.5 per cent HCl was successfully used for treating seed potatoes. Mercuric chloride was employed as an insecticide for cabbage-maggot but cost of material and application prevented extensive use. Dills and Edland (1948). Its use was extended to protection of timbers from termites and in repellents for protection of paper and textile products from insect damage. In gladiolus disease-control studies, Forsberg (1947) found  $\text{HgCl}_2$  effective in the control of *Fusarium* rot and for scab control, although a 14 hour dip could not be safely used on all varieties. Turner *et al.* (1948) annotated list considered  $\text{HgCl}_2$  among other mercurials as "accepted leather fungicides."

*Mercurous Chloride.*—Calomel ( $\text{HgCl}$ ), which is insoluble in water or organic solvents, has been employed in deep, septic wounds and in dressings where proper attention could not be given. Its action as an antiseptic probably depends on its slow change to a more active mercury salt. Although  $\text{HgCl}$  was found ineffective

alone, Hobson (1941) reported the possible use of calomel in protective dusts and dressings for control of sheep maggots, due to its activation by wool and its components.

*Mercury Oxycyanide.*— $\text{Hg}(\text{CN})_2\text{HgO}$ , as an article of commerce, contains about 33 per cent mercuric oxycyanide and 67 per cent mercuric cyanide to eliminate danger of explosion with a flame or by percussion. As an antiseptic, a 1:6000 aqueous solution, pH 9.1, is recommended. Mercuric oxycyanide is almost non-corrosive to instruments and has been used to disinfect surgical instruments, catheters and other equipment which may be damaged by autoclaving. For this purpose, a 0.1 per cent solution is frequently employed. Eye drop prescriptions are sometimes formulated with a one-tenth grain of  $\text{Hg}(\text{CN})_2\text{HgO}$  per ounce.

*Mercuric Cyanide.*— $\text{Hg}(\text{CN})_2$  has found limited use as an antiseptic. The recommended dilution is 1:1000 aqueous solution—pH 6.2. Using 0.05 ml of 1:400 dilution of  $\text{HgCl}_2$  as the standard in a procedure similar to paper disk assay of antibiotics, Herrero (1945) assigned a factor ratio of 1:5, respectively, to  $\text{HgCl}_2$  and mercuric cyanide.

## PHARMACEUTICAL USE OF INSOLUBLE SALTS

As has been suggested many of the mercury salts are insoluble and these have been incorporated in ointment bases for use as antiseptics. Their long use and success in this field is based on the fact that when compounded in this manner they slowly release some of the mercury compound in low concentration and when used like this they can exert their antibacterial action over long periods of time.

Yellow mercuric oxide ointment U.S.P. contains 1 per cent of the mercurial in wool fat and petrolatum. It is usually employed in treating infections of mucous membranes, for example, *pruritus ani*, eye infections, and so forth. *Ammoniated mercury ointment* U.S.P. is a 10 per cent concentration in white wax, wool fat and petrolatum and its chief use is as a skin antiseptic. Metallic mercury is used in two U.S.P. ointment bases. If one incorporated 50 per cent of the metal in a base of wool fat, white wax and petrolatum it is called *Strong Mercurial Ointment U.S.P.* and if 30 per cent is used then it is called *Mild Mercurial Ointment U.S.P.* These two ointments are used in the treatment of syphilis and other skin disorders. In addition to these the National Formulary lists *Strong and Dilute ointments of Mercury Nitrate*, ointment of *Mercurous Chloride* (30 per cent calomel ointment), *Ointment of Red Mercuric Oxide* (10 per cent). The British Pharmacopeia also





(1948), who attempted to show that in order for a mercurial to be an antiseptic, it must be germicidal. A number of papers have appeared in response to the Council's suggestion and from these it is evident that the mercurials under actual conditions of use do act as antiseptics, (Powell, 1948), (Brewer, 1948), (Cromwell, 1949), (Brewer and McLaughlin, 1951).

Much of the recent work to show the ineffectiveness of the organic mercurials is based on the use of thioglycollate medium which we introduced in 1940 with the statement that "the sodium thioglycollate combines with and inactivates most of the mercurials used as preservatives and one is much more likely to obtain growth from contaminated biologicals which are preserved with one of these highly bacteriostatic compounds." (Brewer, 1940). We have shown earlier in Table 6 that it is possible to have organisms present in the body of an animal and that they are not infectious until a very strong reducing agent (BAL) is injected. Even as early as 1891 Tizzoni and Cattani (1891) working with mercuric chloride made the pertinent observation that tetanus spores could be made harmless for animals in a shorter exposure to the drug than was necessary to make them non viable upon subculture. This observation has been confirmed with the more complex organic mercurials, and if one uses thioglycollate medium to recover the organisms the results are more pronounced.

Since non-toxic neutralizers are not available for most of the so-called germicides, tests have not always determined whether

TABLE 11.—SHOWING RELATIVE TOXICITY OF ANTISEPTICS ON *M. PYOGENES* VAR. *AUREUS* AND EMBRYONIC CHICK PERIOSTEAL CELLS\*

<i>Antiseptic</i>	<i>Dilutions Used</i>	<i>Greatest dilution that killed the bacterium used = (Ab)</i>	<i>Greatest concentration in which cells show approximately nor- mal growth = (Ac)</i>	<i>(Ab) (Ac)</i>
Phenol	1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10 × 100	1:200	1:900	0 2
Iodine	1:2, 1:4, 1:6, 1:8, 1:12, 1:15 × 1000	1:2000	1:4000	0 5
Mercurio- chrome	1:4, 1:8, 1:16, 1:20, 1:24, 1:28, 1:32 × 1000	1:16,000	1:32,000	0 5
Metaphen	1:20, 1:40, 1:80, 1:100, 1:120, 1:140, 1:160, 1:180 × 1000	1:100,000	1:180,000	0 6
Merthiolate	1:20, 1:40, 1:80, 1:100, 1:120, 1:140, 1:160, 1:180 × 1000	1:140,000	1:160,000	0 9

\* Buchsbaum, R., and Bloom, W., 1931. The Proceedings of the Society for Experimental Biology and Medicine, 18, 1060-1064.

many of these act as antiseptics because they kill or simply because they inhibit growth. It would be interesting indeed to see all the "germicides" tested with their appropriate neutralizer with as complete data as exist for the mercurials.

Several authors have stated that the ideal antiseptic is one which kills the bacteria without affecting the tissue cells, and they have devised methods of comparing the effects of various mercurials on bacteria and tissue cells both *in vitro* and *in vivo*. One of these which is quite typical is the work of Buchsbaum and Bloom (1931). Results of their experiments are shown in Table 11.

If one adopts the criterion for an ideal antiseptic that it must kill all bacteria at a dilution below that which damages tissue cells, then one would have also an ideal chemotherapeutic agent and the mercurial antiseptics do not fit this requirement (Welch and Hunter, 1940), (Green and Birkeland, 1944), (Salle and Catlin, 1947).

*Merbromin* N.F. (Mercurochrome) was the first of the complex organic mercurials. Chemically it is the sodium salt of di-bromoxymercurifluorescein, it is a dye and the mercury is actually part of the dye molecule. The sodium salt is soluble in water and it is usually employed in a 2 per cent aqueous solution. Since it is non-irritating to tissue it has been widely employed in medicine and some 400 papers have appeared on this one mercurial alone. Since it was the first of these newer organic mercurials it was suggested as a cure for all sorts of diseases and with the usual attendant publications both pro and con, it was soon found that it was not a suitable agent for systemic chemotherapy and it has found its place as a local antiseptic. The Council on Pharmacy and Chemistry in its 1950 N.N.R. states that "Merbromin is a non-irritating, moderately active antiseptic. When applied to the skin, mucous membranes, and wounds, it exerts bacteriostatic action. The 2 per cent aqueous solution of merbromin acts more slowly than iodine tincture—U.S.P., but has more prolonged bacteriostatic effect. The aqueous-alcohol-acetone solution called merbromin surgical solution has more rapid action than the aqueous solution and may be used for preoperative skin disinfection. Merbromin penetrates significantly only into dying or dead tissue."

In discussing reduction of infectivity of certain pathogenic bacteria by Mercurochrome (Brewer, 1948), after a series of experiments on bacteriostasis states that, "the treatment of pathogenic bacteria with 1 per cent solution of "Mercurochrome" impairs the infectivity of these bacteria to such an extent that the chance of infection is practically eliminated."

The inability of Merbromin to kill spores has already been discussed.



As far as the ability of Merbromin and other organic mercurials to act bacteriostatically one has a choice of several laboratory methods. These in no way simulate actual conditions of use but are presented to establish the bacteriostatic values. For the most part the information is similar to that given by Birkhaug (1933), Table 10, based on phenol coefficient methods. More recently Engley (1950) employing a paper disc assay method compared the mercurials and stated that by this technique the mercurials gave zones similar to those obtained in the paper disc assay technique with penicillin and other antibiotics.

TABLE 12. COMPARISON OF ANTIBACTERIAL ACTIVITY OF MERCURIAL ANTISEPTICS BY PAPER DISC ASSAY METHOD\*  
(20 ml plate)

<i>Compound</i>	<i>Dilution of use (per cent)</i>	<i>Nutrient agar inhibition zone (in mm)</i>
Phenyl mercuric borate	0.1	33
Mereresin	0.1	32
Mercarboline	0.1	32
Merthiolate	0.1	32
Mercuriofen	0.1	29
Mercuric iodide	0.1	28
Metaphen	0.2	25
Mercurochrome	2.0	24
Merbak	0.1	23
Merodicein	0.2	18
Mercuric chloride	0.1	18

\* Engley, Frank B., Jr., 1950. Mercurial Compounds as Antiseptics, *Ann. N. Y. Acad. Sci.*, 53, 197.

*Thimerosal* N.F. (Merthiolate) the sodium salt of ethyl mercury thiosalicylic acid as an antiseptic has been thoroughly studied by Jamieson and Powell (1931). They stated that merthiolate has many desirable features chief among these being its solubility in body fluids and water, its low toxicity for man and animals and its uniform bacteriostatic and germicidal action. N.N.R. (1950) states, "Thimerosal is bacteriostatic for many non-sporulating bacteria and is also fungistatic. It is used for disinfecting tissue surfaces. However, this agent, like other organic mercurials presently available, cannot be guaranteed to achieve sterilization, especially when spore-forming organisms are present. It is much less toxic than mercuric chloride. Thimerosal 1:10,000 may be useful as a preservative of biologicals whose protein content is not too high; this concentration, however, does not necessarily prevent growth of micro-organisms in stored liquid plasma." Phenol coefficients as high as 1400 have been reported for this compound, however they are subject to the same criticism as can be made for this method

of testing on the other mercurials. Marks, Powell, and Jamieson (1932) have reported merthiolate to be effective in maintenance of skin antiseptics.

Powell and Culbertson (1950) discussed the assay of antiseptics at different times after application to human skin. Their statement of the problem and their results are worth noting. "Although a vast amount of information has been published concerning the many chemicals intended as antiseptics for human use, it would appear to help very little in answering the final question of practical effectiveness when applied to the human skin. The present authors believe, from many years of experience, that the effectiveness of antiseptics as applied to the human body has never been established clearly, and that the proper specific laboratory tests to reflect degree of utility have not yet been devised. This situation would also seem to be implied by the fact that there exists a long and conglomerate list of tests set up to "prove" or "disprove" the usefulness of proposed antiseptics."

"There is very little information as to what happens to antiseptics once they are applied to tissues. This is in sharp contrast, for example, to the available knowledge dealing with the fate of sulfonamides, antibiotics, etc. after use of these agents. Are the antiseptics fully as active as the result of tube tests indicate, or are they rapidly converted to an inactive form? In this report we have attempted, through preliminary experiments on normal human skin, to learn something of the rate at which the activity of antiseptics is lost when they are painted on the skin."

Tests have been conducted on Merthiolate, Metaphen, Zephiran, and iodine to determine the property of remaining in inactive form after applications to human skin. Assays of washings from treated areas show a decrease of activity with the passage of time. The results suggest that Merthiolate is recoverable by aqueous alcohol but not by aqueous solvents. The methods used did not yield satisfactory results with iodine. It is concluded from these results that all the antiseptics remained in situ for as long as 8 hours in dilutions stronger than those necessary for suppression of staphylococcal growth, since the washings had to be diluted at least 25- to 50-fold in order to obtain growth of the test organisms.

The fact that thimerosal was not a dye like merbromin led to its use as a biologic preservative and it has been widely used for this purpose. The fact that it does not denature proteins and polysaccharides as much as phenol or tricresol (Krueger and Nichols, 1936), has contributed to its success in this field.

*Nitromersol* (Metaphen), the anhydride of 4-nitro-3hydroxy-mercuri-O-cresol, was synthesized as a result of studies of com-

pounds involving a nitro-benzene-mercury complex. The anhydride ring opens—on being dissolved in alkali—to form the hydroxymercury salt. Its germicidal properties initially were reported to be effective particularly against Gram-positive cocci (Raiziss, Severac, and Moetsch, 1930). Metaphen was reported not only effective on other microorganisms but also active against spore-forming bacteria such as the bacillus of anthrax. Such germicidal claims were based on phenol coefficient type tests with such organisms as *M. pyogenes* var. *aureus* and *S. typhosa*. Our intentions are not to develop the many experimental results presented to establish the merits of this compound; these results are discussed in numerous publications and several textbooks.

Briefly, the usual data quote Nitromersol to be 11 times more "germicidal" than bichloride of mercury, and to have a phenol coefficient value of 400 to 2,000 against various microorganisms (Birkhaug, 1933), although reported by Salle and co-workers (1939) as 12.5 to 30.5. Experimental procedures employing the neutralizers, sodium thioglycollate and BAL, have demonstrated the limits of these claims of high germicidal and sporicidal activity: however, Brewer (1950) has demonstrated the ability of nitromersol to show 70 per cent protection against 100 lethal doses of tetanus spores. As previously stated in a study of the practical effectiveness when applied to the human skin, Powell and Culbertson (1950) concluded that metaphen (one of the mercurials studied) remained "in situ" for as long as 8 hours in titers stronger than those necessary for suppression of staphylococcal growth.

New and Non-official Remedies (1950) contains these statements on the actions and use of Nitromersol—NF—Metaphen: "Nitromersol is used only in the form of the sodium salt, which is more germicidal than mercuric chloride when tested on cultures of *M. pyogenes* var. *aureus* and *S. typhosa*. It is non-irritating to mucous membranes or the skin, is without deleterious action on metallic instruments or rubber and is non-toxic. Nitromersol is used in the treatment of gonorrhea and other infections of the eye; for the disinfection of skin, surgical instruments and rubber if no sporulating pathogenic organisms are present." These solutions are recommended as "Use Dilutions": for disinfection of instruments 1:1,000 to 1:5,000: for application to the skin 1:1000 to 1:5000: for ophthalmologic and for urethral irrigation 1:5000 to 1:10,000.

## PHENYLMERCURIC COMPOUNDS

Impairment of the antiseptic activity of inorganic mercury salts by serum, organic matter, and in particular —SH bearing compounds, lead to the introduction of the phenylmercuric salts,



otably acetate, borate and nitrate. The acid radical influenced solubility and other physical properties but did not alter the anti-bacterial activity of the phenylmercuric cation ( $C_6H_5Hg^-$ ). In general, aqueous solutions, buffered with either organic or inorganic acids, are reasonably stable, colorless, odorless and relatively non-corrosive. An excellent presentation on Merphenyl Borate, Nitrate (basic), and Picrate (tincture) is contained in McCulloch's "Disinfection and Sterilization" (1945). The antiseptic action of phenylmercuric nitrate and related compounds was reviewed by Voge (1947).

Aside from their use as medicinal antiseptics, the phenylmercuric compounds have proven useful as economic and industrial preservatives. Phenylmercuric nitrate was reported effective as a bactericide for sterilization of solutions with a chemical agent and heat at  $80^\circ C$ . McEwan and Macmorran (1947) reported 42 medications to dissolve in 0.002 phenylmercuric nitrate. However, Erne and Söderlundh (1946) found phenylmercuric nitrate incompatible with more medicinals than other chemicals tested. Smithers (1945) recommended phenylmercuric nitrate for use with digitalin and ergotinine citrate, if these materials are to be sterilized by heat.

Turner *et al.* (1948) annotated list also contained phenylmercuric acetate and phenylmercuric nitrate as "accepted leather fungicides." Phenylmercuric acetate, 0.03 per cent; phenylmercuric acetate complex, 0.03 per cent; and phenylmercuric octodeconoic acid, 0.2 per cent, were found effective leather fungicides by ALCA method (Cordon *et al.*, 1949). Gran *et al.* (1951) reported phenylmercuric acetate to be considered as one of the most effective disinfectant agents in the pulp and paper industry. A dosage of 0.1 mg per L was found sufficient against all organisms encountered. Microbiological degradation of sodium carboxymethyl cellulose was prevented with 0.1 mg per L of phenylmercuric acetate. Use of phenylmercuric lactate has been mentioned in approach to slime control in the paper mill industry.

As a preservative in antigens phenylmercuric acetate (1:25,000 dilution) was recommended by Williams and Piness (1950). Prevention of mold growth in dye solutions used in color photography was accomplished by the addition of a small "pinch" of phenylmercuric chloride. Beal (1948) found this practice to have no effect on the workings of the dye. For other commercial uses of phenylmercuric derivatives one is referred to a review by O'Brien (1946).

## ETHYLMERCURY PHOSPHATE

When ethylmercury phosphate was used as a saturant for impregnation of dressing material, better bacteriostatic and bactericidal activity was obtained than with  $HgCl_2$  (Kavenoki, 1946).

Several reports have appeared on the use of ethyl mercury phosphate in the control of seed-borne diseases of cereals.

Mertoxol (acetoxymercuri-2-ethyl - hexyl - sulfonic acid) was made by introducing an acetoxy-mercuri grouping into a water soluble derivative of the higher phenols, 2-ethyl-hexyl-phenol. The sulfonic acid radical contributes to the properties of solubility in water, greater penetrability due to reduction in surface tension, and a diminishing effect on the toxicity of the original phenol. It contains approximately 40 per cent mercury organically combined. Mertoxol is reported to be effective against both Gram-positive and negative bacteria, and phenol coefficients of 100 to 500 have been ascribed to this compound. A 1:1,000 isotonic solution of Mertoxol could not be relied upon to kill bacterial spores (Brewer, 1939).

Mertoxol is recommended for general surgical antisepsis and infections of the genito-urinary tract, eye, ear, naso-pharynx, and skin. Excepting the tincture, which is tinted, it does not stain skin or fabric, is non-irritating, and is, like the other mercurials, practically non-toxic in dilutions indicated.

Because of the limitations of the mercurials to kill spores, and since they are inactivated by SH compounds, many workers have tried the addition of organic solvents, wetting agents or other antibacterial products to overcome these criticisms. The first approach was to make these mercurials into tinctures primarily so that they would wet greasy skin and thus might prove more useful.

In an effort to show whether or not the addition of a wetting agent would act synergistically, organisms were mixed with mercurials and, after standing different lengths of time, were subcultured into medium containing a reducing agent. This was done with 3 representative organisms tested against the 6 mercurials with and without addition of sodium lauryl sulfate. In the following tables it will be noted that the mercurial alone was incapable of killing all of the organisms, as was the wetting agent in 0.1 per cent concentration, although a combination of the two was actually germicidal for each organism. When mercurials are combined with surface tension depressants, greater germicidal activity will be obtained than with either agent alone. (Lilley and Brewer, 1949). (See Table 13.)

## GENERAL CONSIDERATIONS

Considering the use of the mercurials from a purely historical view point it is a well recognized fact that they helped prevent sepsis since they were used for this purpose long before the germ theory of disease was established. As has been stated previously

TABLE 13

BACTERICIDAL ACTIVITY OF MERCUROCHROME WITH 0.1 PER CENT SODIUM LAURYL SULFATE  
(0.1 ml subcultures into 15 ml fluid thioglycollate medium and incubated 7 days.)

Solutions tested	<i>Micrococcus pyogenes</i> var. <i>aureus</i> 209				<i>Escherichia coli</i>				<i>Pseudomonas</i> <i>aeruginosa</i>			
	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs
1% Sodium lauryl sulfate	+	+	+	+	+	+	+	+	+	+	+	+
:50 Aqueous Mer- curochrome	+	+	+	+	+	+	+	+	+	+	+	+
:50 Aqueous Mer- curochrome & 0.1 % sodium lauryl sulfate	+	—	—	—	+	—	—	—	+	+	+	—

BACTERICIDAL ACTIVITY OF PHENYL MERCURIC NITRATE WITH 0.1 PER CENT SODIUM  
LAURYL SULFATE

(0.1 ml subcultures in 15 ml fluid thioglycollate medium and incubated 7 days.)

Solutions tested	<i>Micrococcus pyogenes</i> var. <i>aureus</i> 209				<i>Escherichia coli</i>				<i>Pseudomonas</i> <i>aeruginosa</i>			
	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs
1% Sodium lauryl sulfate	+	+	+	+	+	+	+	+	+	+	+	+
:1,500 Phenyl Mer- curic nitrate	+	+	+	+	+	+	+	+	+	+	+	+
:1,500 Phenyl Mer- curic nitrate & 0.1 % sodium lauryl sulfate	+	—	—	—	+	—	—	—	+	—	—	—

BACTERICIDAL ACTIVITY OF MERODICEIN WITH 0.1 PER CENT SODIUM LAURYL SULFATE\*

(0.1 ml subcultures in 15 ml fluid thioglycollate medium and incubated 7 days.)

Solutions tested	<i>Micrococcus pyogenes</i> var. <i>aureus</i> 209				<i>Escherichia coli</i>				<i>Pseudomonas</i> <i>aeruginosa</i> <sup>7</sup>			
	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs	2 10 min	2 30 min	1 hr	2 2 hrs
1% Sodium lauryl sulfate	+	+	+	+	+	+	+	+	+	+	+	+
:500 Aqueous Mer- odicein	+	+	+	+	+	+	+	+	+	+	+	+
:500 Aqueous Mer- odicein & 0.1% sodium lauryl sul- fate	—	—	—	—	+	+	—	—	+	+	—	—

\* Merodicein is the Hynson, Westcott and Dunning brand of monohydroxy-mercuri-  
iodoresorcinsulfonphalein-sodium.



TABLE 13 (CONTINUED)

BACTERICIDAL ACTIVITY OF SODIUM ETHYLMERCURI-THIOSALICYLLATE WITH 0.1 PER CENT SODIUM LAURYL SULFATE

(0.1 ml subcultures in 15 ml fluid thioglycolate medium and incubated 7 days.)

Solutions tested	<i>Micrococcus pyogenes</i> <i>var. aureus</i> 209				<i>Escherichia coli</i>				<i>Pseudomonas</i> <i>aeruginosa</i>			
	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs
1:1% Sodium lauryl sulfate	+	+	+	+	+	+	+	+	+	+	+	+
1:1,000 Sodium ethylmercuri-thiosalicylate	+	+	+	+	+	+	+	+	+	+	+	+
1:1,000 Sodium ethylmercuri-thiosalicylate & 0.1% sodium lauryl sulfate	—	—	—	—	+	—	—	—	+	+	—	—

BACTERICIDAL ACTIVITY OF BICHLORIDE OF MERCURY WITH SODIUM LAURYL SULFATE

(0.1 ml subcultures in 15 ml fluid thioglycollate medium and incubated 7 days.)

Solutions tested	<i>Micrococcus pyogenes</i> <i>var. aureus</i> 209				<i>Escherichia coli</i>				<i>Pseudomonas</i> <i>aeruginosa</i>			
	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs
0.1% Sodium lauryl sulfate	+	+	+	+	+	+	+	+	+	+	+	+
1:1,000 Bichloride of mercury	+	+	+	+	+	+	+	+	+	+	+	+
1:1,000 Bichloride of mercury & 0.1% sodium lauryl sulfate	+	—	—	—	+	—	—	—	+	+	—	—

BACTERICIDAL ACTIVITY OF NITROMERSOL WITH SODIUM LAURYL SULFATE

(0.1 ml subcultures in 15 ml fluid thioglycollate medium and incubated 7 days.)

Solutions tested	<i>Micrococcus pyogenes</i> <i>var. aureus</i> 209				<i>Escherichia coli</i>				<i>Pseudomonas</i> <i>aeruginosa</i>			
	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs	10 min	30 min	1 hr	2 hrs
0.1% Sodium lauryl sulfate	+	+	+	+	+	+	+	+	+	+	+	+
1:500 Nitromersol	+	+	+	+	+	+	—	—	+	—	—	—
1:500 Nitromersol & 0.1% sodium lauryl sulfate	—	—	—	—	+	—	—	—	+	—	—	—

There was indeed the ideal antiseptic test—the ability of these compounds to prevent sepsis under actual conditions of use, with hundreds of years of untreated infected cases acting as controls.

The scientific literature of the last 50 years abounds in efforts to show the limitations of the mercurials. The most severe criticism is that the mercurials can be neutralized by SH compounds found in the body. This criticism can be borne out by laboratory data; however, to suggest that the mercurials cannot act as antiseptics simply because they are easily neutralized by substances found in the body, such as glutathione, is like suggesting that the sulfonamides are not satisfactory chemotherapeutic agents, since they are easily neutralized by *p*-aminobenzoic acid, or that penicillin will not work orally since *E. coli* and other intestinal organisms produce penicillinase which inactivates this antibiotic. It is readily agreed that the inherent toxicity of the mercurials removes them from the realm of usefulness as systemic chemotherapeutic agents and their inability to kill spores prevents their use as germicides for sterilizing instruments. Fortunately the mercurials will act as bacteriostatic agents to both spore forming and non-sporulating organisms and thereby prevent infection as borne out in *in vivo* tests by Fizzoni and Cattani (1891), and later by Brewer and McLaughlin (1951). This work does not suggest that any of the mercurial antiseptics can be depended on to prevent infection by spores in deep puncture wounds, gangrenous areas or other conditions where antitoxin should be relied upon. One should rely on clinical experience in such matters and clinical records bear out the view supported by Lister's original record based on Koch's work that contaminated wounds treated promptly with antiseptics do not suppurate.

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## II

# HEAVY METALS OTHER THAN MERCURY AND SILVER

HEAVY metals other than mercury and silver exert only a feeble germicidal action on bacteria.

Metal salts, notably of lead, zinc, copper, and aluminum are effective chiefly by virtue of their ability to act as astringents. Astringents are agents used locally for their precipitating action on protein material, forming a protective layer of coagulated protein about the infected area, thus aiding in the repair of tissue. The ability of astringent salts in precipitating protein probably accounts for their effectiveness in destroying bacteria. They are frequently associated in their use with antiseptic substances. For example, astringent salts are often used in the form of lotions or washes for local application to wounds, or as contractile agents in large ulcers, etc., where antiseptic and astringent effects are highly desirable.

The ability of metallic salts to promote healing of an infected area would suggest that these agents possess either antiseptic or germicidal powers. This accounts for their early use as astringent agents, especially salts of zinc and aluminum.

Sprowls and Poe (1943a) made a study of the disinfecting powers of a number of astringent salts and official astringent solutions according to modern methods of antiseptic testing. They found that almost all inorganic salts show some degree of astringency, the extent of this action being usually proportional to the concentration of the salt. All inorganic salts may be considered astringents under certain conditions. This is especially true of salts of aluminum, zinc, and lead, which are widely used for this purpose.

An astringent action may be followed by an irritant or a caustic effect, depending upon the concentration of the salt. These are merely degrees of the same process. Therefore, the classification of salts as astringent or otherwise is an arbitrary one.



Sprowls and Poe made a study of the maximum dilution of salts required to kill *Salmonella typhosa* and *Micrococcus pyogenes* var. *aureus* in 10 minutes but not in 5. The Food and Drug Administration method of antiseptic testing was followed. The results are recorded in Table 14. The typhoid organism was found to be more susceptible to the action of the salts than was *M. pyogenes* var. *aureus*. Salts of copper, iron, and tin were found to be fairly effec-

TABLE 14.—MAXIMUM DILUTION OF SALTS REQUIRED TO KILL SALMONELLA TYPHOSA AND MICROCOCCUS PYOGENES VAR. AUREUS IN 10 MINUTES BUT NOT IN 5

Salt	Maximum dilution	
	<i>S. typhosa</i>	<i>M. pyogenes</i> var. <i>aureus</i>
$\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$	1:3	*
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	1:20	1:6
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	1:5	1:5
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	1:300	1:7
$\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$	1:50	1:6
$\text{CuSO}_4$	1:40	—
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	1:200	1:10
$\text{Fe}_2(\text{SO}_4)_3$	1:500	1:30
$\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$	1:3	—
$\text{Pb}(\text{NO}_3)_2$	1:3	—
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	1:5	1:3
$\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	1:3	1:3
$\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$	1:350	1:70
$\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$	1:3	—
$\text{ZnCl}_2$	1:8	—
$\text{ZnI}_2$	1:30	1:10
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1:3	—
Phenol	1:90	1:60

\* Indicates that a saturated solution of the salt failed to kill.

tive against *S. typhosa* but practically without effect against *M. pyogenes* var. *aureus*. The figures show the high concentrations of the salts which generally must be used in order to kill the organisms in a period of 10 minutes. A number of salts were not included in the list because they failed to kill either test organism in the test period.

They concluded that of a total of 24 astringent salts tested (not including mercury and silver) none of them was found to be particularly effective against the test organisms. The results would indicate that much of the literature on the effectiveness of astringent salts is in need of revision.

A number of investigations indicate that the addition of astringent substances to germicidal preparations may have an enhancing effect upon the latter. Sprowls and Poe (1943b) combined phenol, Hexylresorcinol, Merthiolate, and Metaphen with various astringent salts of aluminum, copper, iron, lead, and zinc; and tannic acid.

Solutions for the tests were made by combining sufficient amounts of the inorganic astringents with each of the antiseptic preparations to make the solutions one-tenth molar with respect to the astringent. The tests were performed according to the official F.D.A. method of antiseptic testing.

They found that the astringents were capable of increasing the germicidal power of the antiseptics tested. The effect upon germicidal power, of the addition of the astringent substances to

TABLE 15.—EFFECT OF ASTRINGENT SUBSTANCES WITH PHENOL AGAINST MICROCOCCUS PYOGENES VAR. AUREUS

Combination	pH	Dilution of combination that kills in 10 minutes	Conc. of antiseptic present	Conc. of astringent present	Conc. of antiseptic that kills in 10 minutes	Per cent increase in germicidal efficiency of antiseptic
Phenol + M/10 AlCl <sub>3</sub>	3.3	1:4½	1:90	M/45	1:80	10
+ M/10 Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	3.5	1:7	1:140	M/70	1:80	75
+ M/10 K Alum	3.5	1:4	1:80	M/40	1:80	0
+ M/10 CuCl <sub>2</sub>	3.6	1:7½	1:150	M/75	1:80	90
+ M/10 CuSO <sub>4</sub>	3.6	1:5½	1:110	M/55	1:80	40
+ M/10 FeCl <sub>3</sub>	2.2	1:6½	1:130	M/65	1:90	45
+ M/10 Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	2.3	1:8	1:160	M/80	1:90	80
+ M/10 PbAc <sub>2</sub>	5.0	1:4	1:80	M/40	1:80	0
+ M/10 ZnAc <sub>2</sub>	6.0	1:4	1:80	M/40	1:80	0
+ M/10 ZnCl <sub>2</sub>	5.4	1:4½	1:90	M/45	1:80	10
+ M/10 ZnSO <sub>4</sub>	4.9	1:4½	1:90	M/45	1:80	10
+ M/10 Zn	6.4	1:4½	1:90	M/45	1:80	10
Phenolsulfonate						
+ M/10 Tannic acid	3.5	1:7	1:170	1:70	1:80	75

disinfectants, was more pronounced when a Gram-negative organism was used than when the test organism was Gram-positive. Very small concentrations of the astringents were found to be capable of increasing the bacteriostatic power of some of the antiseptic agents. There appeared to be maximum dilutions for both antiseptic and astringent beyond which no enhancement of inhibiting effect was manifested by combinations of the substances. A typical set of results is shown in Table 15.

*Antagonistic Action of Ions.*—Winslow and Falk (1923a) found that *Escherichia coli* remained viable in distilled water at pH 6.0 without material loss in bacterial numbers for almost 24 hours. At pH 5.0 the decrease in numbers was somewhat greater. The viability decreased as the solution was adjusted to more acid or alkaline conditions.

On the other hand, NaCl in a strength of 0.0145M exerted a favorable action on the viability of *E. coli*. Instead of a drop in the bacterial population after 24 hours, the organisms maintained themselves in undiminished numbers. At a concentration greater

than 0.0145M NaCl, the percentage of living organisms decreased with increasing concentrations of salt.

Calcium chloride behaved in a similar manner. The organisms maintained themselves better in the presence of this salt than in distilled water. A pH of 6 appeared to be the most favorable for maintenance of the organisms. The most favorable concentration appeared to be 0.00145M which would indicate that  $\text{CaCl}_2$  is more toxic than NaCl.

In a later report, Winslow and Falk (1923b) stated that solutions of 0.725M NaCl and above and solutions of 0.435M  $\text{CaCl}_2$  and above exhibited distinctly toxic actions on *E. coli* at all pH concentrations. However, in a solution containing a mixture of both salts in appropriate proportions, an antagonistic action occurred which tended to protect the bacteria against the toxic action of each salt if present alone. This phenomenon is referred to as the antagonistic action of ions.

MacLeod and Snell (1950) reported that zinc was toxic for *Lactobacillus arabinosus* and that its toxicity could be destroyed by manganese. Magnesium, calcium, and strontium also destroyed the toxicity of zinc for the same organism.

On the other hand, the above ions failed to destroy the toxicity of zinc for *Leuconostoc mesenteroides*. They believed that the toxicity of zinc for *L. arabinosus* resulted from the formation of a catalytically inactive zinc protein from this ion and some protein normally activated by manganese, magnesium, calcium or strontium. The latter ions counteracted the toxicity of zinc by displacing zinc from the protein to form a catalytically active metalloprotein.

Abelson and Aldous (1950) reported that nickel, cobalt, cadmium, zinc, and manganese were toxic for *E. coli*. The toxicity of these cations was reduced in the presence of magnesium. If magnesium was not present in the medium, the metals were toxic in very low levels.

A practical application of this phenomenon was the development by Ringer of a physiological salt solution before ionic antagonism was clearly understood. Ringer found that a pulsating heart, perfused with a 0.75 per cent solution of NaCl, was stopped completely. On the addition of 0.0125 per cent  $\text{CaCl}_2$  to the solution, the heart beat was restored but not in a normal manner. On the further addition of 0.01 per cent KCl and a small amount of sodium bicarbonate to adjust the pH of the solution, the heart beat became normal. This solution is known as Ringer's solution.

*Effect of Oxidation-Reduction Systems.*— Guest and Salle (1942) showed that inorganic ferrous and ferric salts, when tested individually, exhibited very little, if any, germicidal activity against



*S. typhosa* and *M. pyogenes* var. *aureus*. However, if an oxidized and a reduced salt, such as ferric and ferrous chlorides, are mixed to form an oxidation-reduction system, a pronounced germicidal action occurs.

The salts cannot be mixed haphazardly but must be combined in certain definite proportions for maximum germicidal activity

TABLE 16.—EFFECT OF FERROUS AND FERRIC SALTS USED SINGLY AND IN COMBINATIONS AGAINST *MICROCOCCUS PYOGENES* VAR. *AUREUS*

Salt	Killing dilution in 10 minutes at 37° C.*
$\text{FeCl}_2$	1:10 failed to kill
$\text{FeCl}_3$	1:25
$\text{FeCl}_2 + 2\text{FeCl}_3$	1:100
$\text{FeSO}_4$	1:10 failed to kill
$\text{Fe}_2(\text{SO}_4)_3$	1:30
$\text{FeSO}_4 + \text{Fe}_2(\text{SO}_4)_3$	1:75

\* Dilutions of the combinations of chlorides are prepared by mixing 1 g mole of  $\text{FeCl}_2$  and 2 g moles of  $\text{FeCl}_3$ . A 1:100 dilution means 1 g of the mixture diluted to 100 ml with distilled water.

The most effective combination of the sulfates is 1 g mole of each salt.

to occur. In the case of the chlorides, the most effective germicidal action occurs when the salts are mixed in the proportion of two moles of ferric chloride and one mole of ferrous chloride. A 1:100 dilution of the two salts mixed in this proportion killed *M. pyogenes* var. *aureus* in 10 minutes at 37°C.

TABLE 17.—EFFECT OF STANNOUS AND STANNIC CHLORIDES USED SINGLY AND IN COMBINATIONS AGAINST *MICROCOCCUS PYOGENES* VAR. *AUREUS*

Salt	Killing dilution in 10 minutes at 37° C.*
$\text{SnCl}_2$	1:40
$\text{SnCl}_4$	1:50
$\text{SnCl}_2 + \text{SnCl}_4$	1:140

\* The most effective combination of the salts was 1 g mole of each.

The sulfates of the two iron salts behave in a similar manner, which indicates that the effectiveness is due to the positive ions. In this case the most effective combination is in the proportion of one mole of each salt. The results are recorded in Table 16.

The salts of tin and manganese used singly and in combinations give similar results (Table 17). It may be seen that stannous and stannic chlorides tested individually are only mildly effective, whereas the two salts mixed in proper proportions exhibit a more pronounced effect.

The germicidal effect is not limited to salts having a common metallic ion. Salts of different metallic cations and nonmetallic anions may be combined to give the same general effect. The important consideration is that an oxidation-reduction system must be established in order to obtain an increase in the germicidal potency of the solution (Table 18). It may be seen that in every case, a mixture of two salts, one in a higher state of oxidation than the other, resulted in an increased germicidal effect over that of the salts tested individually.

TABLE 18. — EFFECT OF VARIOUS METAL SALTS TESTED SINGLY AND IN COMBINATIONS AGAINST *MICROCOCOCCUS PYOGENES* VAR. *AUREUS*

Salt	Killing dilution in 10 minutes at 37° C.*
FeCl <sub>2</sub> + SnCl <sub>4</sub>	1:130
FeCl <sub>3</sub> + SnCl <sub>2</sub>	1:120
MnSO <sub>4</sub>	1:10 failed to kill
MnSO <sub>4</sub> + SnCl <sub>4</sub>	1:120
MnSO <sub>4</sub> + Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	1:100

The increased killing power of the combinations is essentially a function of the positive heavy metal ions. A mixture of sodium sulfite and sodium sulfate exhibited no increased action over that of the same salts tested individually. The results indicate that the phenomenon is a general one. There was no exception to the rule that a mixture of two heavy metal salts, one in a higher state of oxidation than the other, increased markedly the germicidal efficiency of the same salts when tested singly.

*Effect of O-R Systems on Inorganic Germicides.*—The addition of an appropriate metallic salt to an inorganic germicide, *e.g.*, ferrous sulfate to mercuric chloride, to produce an oxidation-reduction system, resulted in a greater effectiveness of the latter. The addition of another oxidation-reduction system, such as a mixture of ferrous and ferric sulfates, to the ferrous sulfate-mercuric chloride combination, increased still further the efficiency of the mercuric chloride.

In like manner, Salle (1945) found that iodine killed *M. pyogenes* var. *aureus* in a dilution of 1:20,000 and *S. typhosa* in a dilution of 1:17,500 in 10 minutes at 37°C. Iodine dissolved in a 1:3000 dilution of an oxidation-reduction solution (composed of a mixture of one mole each of manganous sulfate and ferric sulfate) killed *M. pyogenes* var. *aureus* in a dilution of 1:80,000 and *S. typhosa* in a dilution of 1:60,000 (Table 19).

*Effect of O-R Systems on Organic Germicides.*—Salle and Guest (1944) found that the addition of an appropriate metallic salt such as ferric chloride to the organic germicide phenol, to produce an oxidation-reduction system, increased greatly the effectiveness of the latter. The addition of another oxidation-reduction

TABLE 19.—EFFECT OF VARIOUS METAL SALTS ON INORGANIC GERMICIDES  
AGAINST *MICROCOCCLUS PYOGENES* VAR. *AUREUS*

<i>Germicide</i>	<i>Killing dilution in 10 minutes at 37° C.</i>
HgCl <sub>2</sub>	1:16,000
HgCl <sub>2</sub> + FeSO <sub>4</sub>	1:40,000 HgCl <sub>2</sub>
AgNO <sub>3</sub>	1:100
AgNO <sub>3</sub> + 2Fe(NO <sub>3</sub> ) <sub>3</sub> + FeSO <sub>4</sub>	1:800 AgNO <sub>3</sub>
Iodine	1:20,000
Iodine + 1:3000 mixture of Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> + MnSO <sub>4</sub>	1:80,000 iodine
Iodine + 1:100 mixture of Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> + MnSO <sub>4</sub>	1:450,000 iodine

system, such as a mixture of ferrous and ferric chlorides, to the ferric chloride-phenol combination, and some glycerin to stabilize the preparation, increased still further the efficiency of the phenol (Table 20).

TABLE 20.—EFFECT OF ADDITION OF METAL SALTS ON GERMICIDAL EFFICIENCY OF  
PHENOL AGAINST *MICROCOCCLUS PYOGENES* VAR. *AUREUS*

<i>Germicide</i>	<i>Killing dilution in 10 minutes at 37° C.</i>
Phenol	1:100
Phenol	1. 25 gm
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> · 5H <sub>2</sub> O	3. 25 gm
Glycerol	50 ml
Distilled water, to make 500 ml	1:1800
Phenol concentration 1:400	
Phenol	1. 25 gm
FeCl <sub>3</sub> · 6H <sub>2</sub> O	3. 60 gm
FeCl <sub>2</sub>	1. 70 gm*
FeCl <sub>3</sub> · 6H <sub>2</sub> O	7. 20 gm
Glycerol	50 ml
Distilled water, to make 500 ml	1:4500
Phenol concentration 1:400	

\* The mixture of ferrous and ferric chlorides represents another oxidation-reduction system.

In like manner, an aqueous solution of cresol killed *M. pyogenes* var. *aureus* in a dilution of 1:300 in 10 minutes at 37°C. The addition of ferric chloride to the cresol, in the proportion of 1 gram mole of each, increased the killing dilution of cresol from 1:300 to 1:4000 or a 13-fold increase in its efficiency. The addition, to the



solution of cresol and ferric chloride, of another oxidation-reduction system, composed of a mixture of ferrous and ferric chlorides in the proportion of 1 gram mole of ferrous chloride to 2 gram moles of ferric chloride, increased the killing dilution of cresol from 1:300 to 1:12,000 or a 40-fold increase in its efficiency (Table 21).

TABLE 21.—EFFECT OF ADDITION OF METAL SALTS ON GERMICIDAL EFFICIENCY OF CRESOL AGAINST *MICROCOCCUS PYOGENES* VAR. *AUREUS*

<i>Germicide</i>		<i>Killing dilution in 10 minutes at 37° C.</i>
Cresol		1:300
Cresol	1 45 gm	
FeCl <sub>3</sub> 6H <sub>2</sub> O	3 60 gm	
Distilled water, to make	500 ml	1:4000
Cresol concentration	1:345	
Cresol	1 45 gm	
FeCl <sub>3</sub> 6H <sub>2</sub> O	3 60 gm	
FeCl <sub>2</sub>	1 70 gm*	
FeCl <sub>3</sub> 6H <sub>2</sub> O	7 20 gm	
Distilled water, to make	500 ml	1:12,000
Cresol concentration	1:345	

\* The mixture of ferrous and ferric chlorides represents another oxidation-reduction system

Similar results were obtained in studies on Hexylresorcinol. The addition of ferric chloride to Hexylresorcinol in the proportion of 1 gram mole of each, increased the killing dilution from 1:6000 to 1:24,000. The results are recorded in Table 22.

TABLE 22.—EFFECT OF ADDITION OF FERRIC CHLORIDE ON GERMICIDAL EFFICIENCY OF HEXYLRESORCINOL AGAINST *MICROCOCCUS PYOGENES* VAR. *AUREUS*

<i>Germicide</i>		<i>Killing dilution in 10 minutes at 37° C.</i>
Hexylresorcinol		1:6000
Hexylresorcinol	1 gm	
FeCl <sub>3</sub> 6H <sub>2</sub> O	3 gm	
Distilled water, to make	1500 ml	1:24,000

Gershenfeld and Greene (1950) tested a number of oxidation-reduction systems in combination with the organic iodine complex N,N,N',N'-ethylene diamine tetra-acetic acid KI<sub>3</sub>.

The most effective oxidation-reduction system was composed of equimolar concentrations of FeCl<sub>3</sub> and MnCl<sub>2</sub>. The killing dilution of the test disinfectant against *M. pyogenes* var. *aureus* was 1:3000. In the presence of a 1:40 dilution of the oxidation-reduction system the killing dilution was 1:15,000. Against *S. typhosa* the killing dilution was 1:4000 and in the presence of a

:60 dilution of the O-R salts the efficiency was increased to :60,000.

The *in vitro* antibacterial efficiency of N,N,N',N'-ethylene diamine tetra-acetic acid  $KI_3$  complex was increased when equimolar proportions of an  $FeSO_4-Fe_2(SO_4)_3$  oxidation-reduction system were added to it. The test disinfectant was potentiated from :3000 to 1:10,000 against *M. pyogenes* var. *aureus*, and from :4000 to 1:40,000 against *S. typhosa*.

It may be concluded that the addition of an appropriate metallic salt to the organic germicides phenol, cresol, and Hexylresorcinol to produce oxidation-reduction systems resulted in a great increase in their efficiencies against *M. pyogenes* var. *aureus*. The addition of another oxidation-reduction system to the germicides increased still further the efficiency of the compounds. It was found that maximum efficiency occurred only if the salts and germicides were mixed in certain definite proportions. The phenomenon applies to oxidation-reduction systems composed of organic germicides with inorganic salts as well as to combinations of inorganic metallic salts.

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## 12

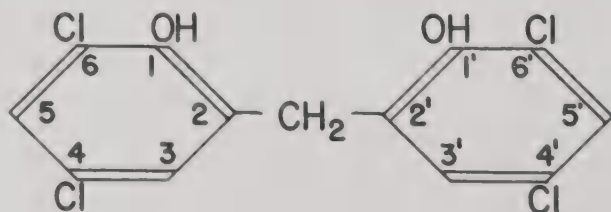
# THE BIS-PHENOLS

### INTRODUCTION

BECAUSE certain members of this chemical group possess high bacteriostatic or fungistatic powers and, for that reason, have found important practical applications, the bis-phenols as a group deserve a prominent place in any compilation of germicides, antiseptics, fungicides, etc.

As the name indicates, bis-phenols are composed of two phenolic bodies, attached together by various linkages. A large number of such compounds are known and more could be synthesized, as there exists the possibility of many variations. The compounds may be symmetrical, the two phenols being alike, or asymmetrical, two different phenols being connected. The linkages may consist of a direct bond from carbon to carbon or there may be a connecting atom, such as sulfur or oxygen, or groups of various types, the most important being alkylene, especially methylene,  $-\text{CH}_2-$ . An important factor is the position of the linkage to the phenolic hydroxyl groups, whether ortho, meta, or para.

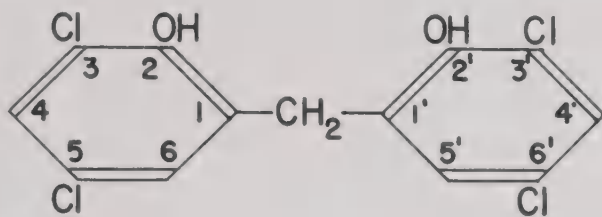
The nomenclature is somewhat involved because two ways of numbering the substituents are in use. If positions 1 and 1' are occupied by the hydroxyl groups, the compound of the following configuration:



is to be called 2,2'-methylenebis-(4,6-dichlorophenol). This is the preferred name in conformity with Chemical Abstracts.



If the numbering starts with the linkage being in positions 1 and 1', the compound is named bis(3,5-dichloro-2-hydroxyphenyl)



methane or 2,2'-dihydroxy-3,5,3',5'-tetrachlorodiphenylmethane. For asymmetrical compounds, only names of the last type can be used.

## HISTORICAL DEVELOPMENT

The antibacterial action of bis-phenols was first demonstrated by Bechhold and Ehrlich (1906). These investigators found that the linkage of two phenols, either directly or by means of an alkylene-group, increases greatly the bacteriostatic and bactericidal potency of the phenols themselves. Particularly effective were 4,4'-3,6'-tetrachloro- and tetrabromo-o-o'-biphenol.

Not much was done with bis-phenols, especially halogenated ones, until chemists at the Bayer plant of I. G. Farbenindustrie A. G. in Germany made an intensive investigation of this type of compounds, beginning in the year 1927, in connection with their search for new mothproofing agents. Weiler, Wenk and Stötter (1929) described the synthesis of 2,2'-methylenebis-(p-halogenated phenols) and during the years following this first patent, a large number of various types of bis-phenols were prepared. Besides their suitability as mothproofing agents—one of the chlorinated bis-phenols, the sodium salt of 5-chloro- $\alpha,\alpha$ -bis(3,5-dichloro-2-hydroxyphenyl)-o-toluene sulfonic acid, was introduced commercially under the tradename Eulan CN—their antibacterial and antifungal properties were mentioned; however, no attention was paid to the fact that maximum activity was dependent on definite structural requirements. Most of the substances described were bis-phenols with methylene and other alkylene or with aryl-alkylene bridges; compounds with sulfur (Wesenberg and Muth, 1932) and oxygen linkages (Muth and Wesenberg, 1936) were also patented as useful for disinfecting and preserving purposes.

While these studies were going on in Germany, some work on the antibacterial properties of similar bis-phenols was published in this country. Dunning and his associates (1931) prepared a

series of 2,2'-thiobis-phenols and showed that the halogenated members were quite bactericidal against *M. pyogenes* var. *aureus*. Moness *et al.* (1932) made an extensive study of the germicidal activity of aromatic sulfides, including 4,4'-thiobis-phenol which was not very potent. Harden and Reid (1932) synthesized a large number of bis-phenols where the linkage was in para position to the hydroxyl groups. They claimed fairly high activity against *M. pyogenes* var. *aureus* for some of their compounds where the phenols were connected by alkylene groups with four to six carbon atoms. Unfortunately, the preparation of such compounds in pure form is very difficult. Harden and Brewer (1937) synthesized a series of brominated 2,4'- and 2,2'-methylenebis-phenols and found that their monosodium salts were active against *M. aureus* at high dilutions. Harden and Brewer considered these bis-phenols of particular interest because, in their experience, they were the first phenolic compounds which retained their antibacterial activity at the alkaline pH of the sodium salt solutions.

While these references show that certain series of bisphenols possessed considerable activity against bacteria and fungi, nothing could be found in the literature in regard to practical applications of any of the bis-phenols. Beginning in 1937, this group of chemicals, especially of halogenated 2,2'-methylenebis-phenols, was subjected to a thorough investigation in the Givaudan Corp. laboratories and two compounds of these series gained important commercial applications. One is 2,2'-methylenebis-(4-chlorophenol), first prepared by Weiler *et al.* (1929) by condensation of p-chlorophenol with formaldehyde in presence of sulfuric acid, and later by an improved method by Gump and Luthy (1943). It is known under the tradenames G-4<sup>(R)</sup> and Preventol<sup>(R)</sup> G.D., and under its generic name dichlorophene. While active against bacteria, its special merit lies in the field of mildew proofing. The other compound is 2,2'-methylenebis-(3,4,6-trichlorophenol), synthesized by Gump (1941) from 2,4,5-trichlorophenol and formaldehyde, and named G-11<sup>(R)</sup> or hexachlorophene. The outstanding usefulness of G-11 and similar 2,2'-methylenebis-phenols is for the preparation of antiseptic soaps (Kunz and Gump, 1942; Traub *et al.*, 1944; Gump, 1945; Price and Bonnett, 1948; and others). Bis-phenols of related configuration, such as halogenated 2,2'-thiobis-phenols (Kunz, Luthy, and Gump, 1944; Shumard, Beaver, and Hunter, 1953), halogenated o-o'-diphenols (Gump, 1944), and halogenated asymmetrical 2,2'-dihydroxydiphenylmethanes (Moyle and Wolf, 1952a, b), are also suitable as ingredients for antiseptic soaps.

The thiobis-phenols found renewed interest in Germany where Pfleger, Schraufstatter and their colleagues (1949) studied a large

number of them *in vitro* in regard to their bacteriostatic and fungistatic properties. The most effective compounds were 2,2'-thiobisphenols with one and two halogens in each ring. Based on these findings, Richter (1950) investigated 2,2'-thiobis-(4-chlorophenol), D-25', for the clinical treatment of fungus infections, claiming excellent results.

Brief mention should be made of the work by Kuhn, Birkofer, and Möller (1943) on salicil (2,2'-dihydroxybenzil). These investigators who synthesized it for the first time showed that salicil and especially dibromosalicil (5,5'-dibromo-2,2'-dihydroxybenzil) were very potent bacteriostats against *M. pyogenes* var. *aureus*. Clinical reports from Germany indicated that dibromosalicil was valuable as a chemotherapeutic agent, being similar in its action to the sulfonamides. However, Schales and Suthon (1946), confirming the antibacterial activity of dibromosalicil, demonstrated that much of it was lost in presence of serum protein; accordingly, the compound was not effective for the systemic treatment of infectious diseases on clinical trials.

## CORRELATION OF CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY

The relationship between chemical configuration and antibacterial or antifungal activity can be discussed here only briefly.

Bechhold and Ehrlich (1906) observed already that certain halogenated bis-phenols were much more bacteriostatic and bactericidal than the monophenols from which they were derived. This, apparently, is true for all halogenated 2,2'-bis-phenols.

These investigators stated that the linkage of two phenolic rings by -CO- or -SO<sub>2</sub>- diminishes the disinfecting power. However, it might be of interest to mention that bis-(4-hydroxyphenyl) sulfone, which is somewhat related in structure to sulfanilamide and which shows little activity *in vitro*, was able to exert a chemotherapeutic effect *in vivo* on experimental brucellosis in guinea pigs (Menefee and Porton, 1939) and on mice infected with gonococci (Levaditi and Vaisman, 1937).

Bechhold and Ehrlich had also found that the introduction of chlorine or bromine into phenols increases the antibacterial potency; the more halogens are present, the more effective are the compounds. The bis-phenols show a similar pattern in regard to gram-positive organisms; the superiority of the higher chlorinated members of a series becomes particularly evident when tested in presence of soap against *M. pyogenes* var. *aureus* (Kunz and Gump, 1942, 1950).



In the case of gram-negative bacteria (Cade, 1944) and fungi (Marsh and Butler, 1946; Marsh *et al.*, 1949), the most effective compounds are the ones which contain one halogen in 4-position of each ring. Increased halogenation always leads to lower potency. On the other hand, compounds with no halogens are weak against practically all species of micro-organisms.

Besides the requirement that the bis-phenols must contain halogen, another factor is of prime importance. The phenols must be linked in ortho (2,2'-) positions to the hydroxyls to obtain maximum activity. The 3,3'-linkage is less effective (Gump and Cade, 1952) and 4,4'-positions of the bond are the least desirable ones (Marsh *et al.*, 1946, 1949; Pfleger *et al.*, 1949).

While the type of the linkage is of minor significance, it should be small enough (carbon to carbon, methylene, sulfur, oxygen, etc.) so that it does not keep the hydroxyls too far apart. A chelated structure or hydrogen bondage between the two hydroxyl groups may be responsible for the high activity of the halogenated 2,2'-bis-phenols (Chiddix *et al.*, 1952) and perhaps explain the great difference in the ionization constants of the hydroxyls. As example, we cite the values reported by Shumard *et al.* (1953) for 2,2'-thiobis-(4,6-dichlorophenol),  $pK_1$  being 4.82 and  $pK_2$  10.50.

## PHYSICAL, CHEMICAL AND PHARMACOLOGICAL PROPERTIES

As we have just enumerated, only bis-phenols which contain halogen and which are linked in the positions adjacent to the hydroxyl groups are highly effective against bacteria and fungi. For that reason, our further discussion will be mainly restricted to this type of bis-phenols which are of interest in view of their biological activity.

The halogenated 2,2'-bis-phenols are colorless, crystalline, non-volatile, stable substance, usually melting in the range from 100° to 200°C. In the pure state, they are practically odorless. Their aqueous solubility is very low; their alkalimetal salts are soluble in water, to varying degrees. Tween<sup>(R)</sup> 20 and Tween 80 were recommended as solubilizing agents to increase the solubility of G-11 in water (Gregg and Zopf, 1951). The compounds containing four and more halogens in the rings form only monoalkali-metal salts (Gump, 1945), even if an excess of alkali is present. Whether the dihalogenated bis-phenols behave in the same manner or whether both hydrogens of the hydroxyl groups may be replaced by metals, has not been established with certainty.

The compounds are soluble in alcohols, glycols, ketones, esters and ethers, slightly soluble in aromatic hydrocarbons and practically insoluble in petroleum solvents. Like other phenols, they darken on exposure to bright light, especially in alkaline media such as soaps.

They also give typical color reactions with ferric chloride and with Folin-Ciocalteu phenol reagent which are useful for their quantitative determinations (Larson, 1951; Reissmann, 1951). Another colorimetric method is based on the formation of red dyes with 4-aminoantipyrine in the presence of potassium ferricyanide and dilute sodium carbonate (Gottlieb and Marsh, 1946). Further analytical procedures suggested specifically for 2,2'-methylenebis(4-chlorophenol) are based on the formation of an intense yellow color upon the treatment of its alkaline solution with sodium hypochlorite (Shiraeff, 1945) and on its oxidation with alkaline potassium permanganate (Jenkins *et al.*, 1946). Here, the chlorine is liberated in form of ionized chloride which can be determined in the usual manner.

The bis-phenols form esters and ethers, and undergo the usual reactions of nitration, sulfonation, halogenation, if reactive hydrogens are available in the rings.

Toxicity to the host, besides that to the micro-organism, is a factor to consider if the compounds are used on humans or animals. It does not necessarily follow the increase or decrease respectively of the antibacterial or antifungal activity. In general, the bis-phenols are less toxic and less irritating than the corresponding monophenols.

The tissue toxicity of 2,2'-methylenebis-(4-chlorophenol), G-4, in relation to its bactericidal activity *in vivo*, was studied by Paulding and Bondi (1947) who showed a favorable infection-prevention toxicity index for this substance. The index for the related 2,2'-methylenebis(3,4,6-trichlorophenol), G-11, (not reported in the paper) was very close to that of G-4. On the other hand, Price and Bonnett (1948) found G-11 and G-5<sup>(R)</sup>, 2,2'-methylenebis(4,6-dichlorophenol), very toxic to dogs when given intravenously, doses as little as 5 mg./kg. of body weight usually causing death.

Not much has been published in regard to the oral toxicity of the bis-phenols in general. Bechhold and Ehrlich had noted that the halogenated bis-phenols which they had tested are less toxic than the halogenated mono-phenols. They also stated that the toxicity of phenols is increased as the numbers of halogens in the ring increases. A similar situation exists in regard to the 2,2'-methylenebis-phenols. Florestano (1949), who had studied the

toxicity and tuberculostatic activity of a fairly large number of such bis-phenols, found that the oral toxicity for mice increased from two to four to six chlorines in the rings, the latter substance (G-11) being the most toxic one, showing a  $LD_{50}$  of 80 mg./kg. for mice, but also the most active one. Nickerson (1950) established for G-11a  $LD_{50}$  of 180 mg./kg. in mice. A higher value, 300 mg./kg. has been reported as minimum lethal dosis for guinea pigs (Gump, 1945).

Richter and Schraufstatter (1950) also showed that the bis-phenols become more toxic with an increased number of halogens; they reported that 2,2'-thiobis (4,6-dichlorophenol) was about 3 times as toxic on oral and intraperitoneal administration to experimental animals as 2,2'-thiobis (4-chlorophenol). The figures given by Shumard *et al.*, (1953) indicate a much lower toxicity for 2,2'-thiobis-(4,6-dichlorophenol), Actamer<sup>(R)</sup>, than that found by Richter and Schraufstatter.

Of greater significance than the oral toxicity of the bis-phenols is their effect on the skin, as the preponderant applications are topical. It became apparent that the halogenated bis-phenols are not irritating to the skin, and very infrequently produce allergic reactions. Especially notable in this respect is G-11 as was shown by Traub *et al.*, (1945); Udinsky, (1945) and others. Schwartz (1950), using his standard patch test procedure, made the interesting observation that a deodorant soap containing 2 per cent G-11 and 1 per cent perfume produced fewer irritating reactions and less sensitization than the same soap without G-11 and perfume.

TABLE 23

Abbreviated name	Generic name	Name based upon structure
G-4	Dichlorophene	2,2'-methylenebis(4-chlorophenol) bis(5-chloro-2-hydroxyphenyl)methane
G-5	Tetrachlorophene	2,2'-dihydroxy-5,5'-dichlorodiphenylmethane 2,2'-methylenebis(4,6-dichlorophenol) bis(3,5-dichloro-2-hydroxyphenyl)methane 2,2'-dihydroxy-3,5,3',5'-tetrachlorodiphenylmethane
G-11	Hexachlorophene	2,2'-methylenebis(3,4,6-trichlorophenol) bis(3,5,6-trichloro-2-hydroxyphenyl)methane 2,2'-dihydroxy-3,5,6,3',5',6'-hexachlorodiphenylmethane
G-5-S	Bithionol	2,2'-thiobis(4,6-dichlorophenol) bis(3,5-dichloro-2-hydroxyphenyl)sulfide 2,2'-dihydroxy-3,5,3',5'-tetrachlorodiphenylsulfide
G-11-S		2,2'-thiobis(3,4,6-trichlorophenol) bis(3,5,6-trichloro-2-hydroxyphenyl)sulfide 2,2'-dihydroxy-3,5,6,3',5',6'-hexachlorodiphenylsulphide



## BIOLOGICAL PROPERTIES OF BIS-PHENOLS

*Nomenclature.*—As mentioned above, some confusion exists in the literature because of different nomenclatures used by different authors in describing the same compound. Therefore, in order to make the material in this chapter consistent, and to make it as easy for the reader as possible, we present here (Table 23) a correlative description of the names used for the individual bis-phenols which are to be discussed in the pages to follow. We will then use to a large extent hereafter in the text the simplest designation (*e.g.*, G-4; G-5; G-5-S; G-11; etc.)

## GERMICIDAL ACTIVITY

*Phenol-coefficients.*—The early literature shows the germicidal potencies of the bis-phenols to be quite high. It is now known that these figures are incorrect, and that the true phenol-coefficients are considerably lower. The reason for this difference is due to the fact that these compounds possess very high bacteriostatic powers, and, as occurred with the mercury compounds, the halogens, and the quaternary ammonium germicides, etc., the influence of this property on the F.D.A. phenol-coefficient test procedure results was not recognized until recently. Now, however, by using proper antitoxins in the subculture media it is found that the true 100 per cent kill end-points (the figures on which the phenol-coefficients are based) for each of these types of materials are much lower than as originally presented.

Taking G-11 for example, it was originally reported to have a coefficient of 185 against *M. pyogenes* var. *aureus* at 37°C (Gump 1941). Later on, using the Cade-Halvorson (1934) plate-count modification of the F.D.A. test, this figure was reduced to around 25 (Cade, 1944) when it was learned that agar plates showed colony growths (even quite high counts) at the same dilutions where the broth tubes of the standard F.D.A. procedure produced minus (—) results. Later Cade (1948), after it was found that normal horse serum served as a good, though not 100 per cent effective, antidote for G-11, by applying this fact to the test procedure, showed the coefficient to be still lower (around 40). It is not outside of the realm of probability that this figure may yet go still lower when, and if, a better antidote is found and used.

It should be recognized in this connection that the solubility of G-11 (and other bis-phenols as well) in water is extremely low, so a true coefficient of it is unobtainable. It must therefore be put into solution by the use of another solvent, or by making an alkali

salt, or by employing a suitable emulsifying agent. Naturally, different results will be obtained depending upon which method is used. In the above data, and in the majority of our own work, we used N/2 alcoholic potassium hydroxide (1.1 mol.) as the solubilizing agent.

Thus, taking into consideration the above facts, it is readily seen why a single numerical value cannot be given for the phenol-coefficient of any of these 2,2'-bis-phenols, whose microbiological activities are based primarily upon their marked bacteriostatic properties. This is due in part to the fact that kill end-points are not sharp, and to the fact that no 100 per cent effective antidote is known at the present time. Serum, although effective to a certain degree, is not 100 per cent efficient. We therefore present here the following general statements as to the germicidal powers of these substances, rather than attempting to list them as specific figures.

*M. pyogenes* var. *aureus* Phenol-coefficients.—All of the practical bis-phenol compounds as used to date, including G-4, G-5, G-5-S, G-11, and G-11-S have coefficients at 20°C in the range around 15 to 40. That means that their 100 per cent kill end-points, based upon the F.D.A. phenol-coefficient procedure, using serum as an antidote, are at dilutions of around 1 to 1000 to 1 to 2500. To calculate closer than that would be more or less meaningless, since the phenol-coefficient test procedure is not more accurate than that. On the same basis as above, the coefficients at 37°C for the respective compounds range between 15 and 40 (or 1 to 1500 to 1 to 4000).

*S. Typhosa* Phenol-coefficients.—For *S. typhosa* the coefficients vary slightly less, since this organism does not respond to bacteriostasis to the degree that *M. pyogenes* var. *aureus* does. G-4 is the most effective compound, with a coefficient of around 25 to 50 (1 to 2000 to 1 to 4000). G-5, G-5-S, and G-11 are about equal but less potent, namely with a coefficient of around 5 to 15 (1 to 500 to 1 to 1500). We have no data on G-11-S, but it would be expected to be close to that of G-11.

Similarly, G-4 has the highest coefficient at 37°C, it being around 25 to 50 (1 to 2000 to 1 to 4000), with the others being closely identical in the range of 5 to 15 respectively (1 to 400 to 1 to 1200).

In this connection one should keep in mind that the phenol-coefficient figures refer to 100 per cent kill results. Compounds with extremely high bacteriostatic properties, like the bis-phenols, produce effective practical germicidal action which, although not 100 per cent, kills over 99.99 per cent of the organisms contacted when much less of the medication is used (sometimes even with one-half

the amount that is required to bring about the 100 per cent kill). Practical phenol-coefficients might be calculated on this basis provided the data are properly qualified. For example, under certain conditions of test, G-11 requires a 1 to 1000 dilution to bring about a 100 per cent kill, while a 99.99 per cent kill would be obtained by a 1 to 2500 dilution. These facts have practical implications and therefore are mentioned here.

*Activity Against Other Organisms.*—So far we have been dealing principally with biological activities against *M. pyogenes* var. *aureus* and *S. typhosa*. Space does not permit giving details of similar data on all other types of organisms. A table of data covering some of the more or less common ones follows. In general, the bis-phenols are more active against the gram-positive organisms than against the gram-negative ones. No doubt this is due, in some degree, at least, to their bacteriostatic properties which are effective against the former more than the latter.

The following tables of data, taken from Sindar Technical Bulletins (48-3, June 1952; and H-4, December 1952) show some of these results:

TABLE 24.—BACTERICIDAL PROPERTIES OF G-4

Organism	Concentration to give growth in <i>F.D.A.</i> broth in 5 minutes but not in 10 minutes	
	20 C	37 C
<i>Corynebacterium diphtheriae</i> (avirulent)	1-9,000	1-30,000
<i>Pseudomonas aeruginosa</i>	1-1,000	1-3,000
<i>Salmonella paratyphi</i> A	1-20,000	1-55,000
<i>Shigella dysenteriae</i> (Flexner)	1-7,000	1-14,000
<i>Neisseria gonorrhoeae</i>	1-35,000	

*Bactericidal Properties of G-11*

<i>C. diphtheriae</i>	1-40,000	1-70,000
<i>Ps. aeruginosa</i>	1-1,000	1-1,100
<i>S. paratyphi</i> A	1-1,000	1-30,000
<i>Sh. dysenteriae</i> (Flexner)	1-1,000	1-3,000
<i>Str. hemolyticus</i> (Lancefield Group A)	1-45,000	1-90,000
<i>N. gonorrhoeae</i>	1-50,000	
<i>B. ammoniagenes</i>	1-2,000	1-7,500

*Effect of Organic Matter.*—As is the case with most germicides, the bis-phenols lose appreciable activity when in the presence of organic matter such as body fluids, pus, serum, albumin, milk, etc. For this reason it has been claimed (Price and Bonnett, 1948) that their efficacy as skin disinfectants, when applied to open wounds, is questionable. This assumption is correct when rapid action is required. However, when long time contact (several hours) is permitted, then it appears that the presence of serum is not too



detrimental from a practical use standpoint, as indicated by the following experimental data from the Sindar laboratories:

TABLE 25

Amount tested		Results* after contact vs. <i>M. pyogenes</i> var. <i>aureus</i> 37° C for			
Bis-phenol	Serum	5 min	3 hours	24 hours	48 hours
G-11	1 1000 in 80% serum	7000	4000	2500	2000
"	50%	6500	550	0	0
"	10%	2500	0	0	0
"	0%	0	0	0	0
G-11	1 2500 in 80% serum	10000	5000	5000	4500
"	50%	10000	8000	1200	0
"	10%	3500	2000	x	0
"	0%	0	0	0	0

\* Test procedure: mixture allowed to stand for times noted, after which a standard loopful was transferred to nutrient agar, incubated 48 hours, and colonies present counted. Figures above 4000 are estimated, as it is impossible to count them accurately.

Thus a statement, that bis-phenols are inactivated by serum, should be qualified as to under what conditions of use, in order to be correct.

*Activity in the Presence of Soaps and Other Detergents.*—A most unique characteristic property of hexachlorophene, and certain other bis-phenols closely related structurally to it, is the fact that it retains sufficient of its germicidal (and also bacteriostatic) powers, when in the presence of soap, to allow it to have useful practical applications (Kunz and Gump, 1942). Most other phenols lose the greater part of their germicidal activity under similar conditions. Chlorine compounds and the quaternaries likewise lose the majority of their effectiveness when compounded with soap. Certain mercury compounds do not, and therefore may serve as the basis for germicidal soaps. However, they have other drawbacks, such as high toxicity, irritation, cumulative poisoning effects, etc., which mitigate against their daily use.

When discussing germicidal soaps, or making comparisons of various soaps containing ingredients assumed to produce germicidal action, one must be certain, or clear, as to their usage definitions. The term, germicidal soap, has become badly misused and refers to so many different sets of conditions, or end results, that the scientific, as well as trade literature, is very ambiguous and somewhat misleading in places. Although hexachlorophene in soap produces a mixture as active germicidally as any other practical compound applicable for the same purpose, and is superior to most other materials used or suggested (*e.g.*, phenols, cresols, quaternaries, chlorine, silver and its salts, mercury compounds, etc.) it

nevertheless does not produce a truly germicidal soap based upon defining the latter as a product which brings about a 100 per cent killing of the test-organism in a short (usually 5 to 10 minutes) time interval at the temperature of use (room temp. to body temp. 25°C to 37°C).

The following table of experimental data (Givaudan laboratories) indicates the general relative activity of hexachlorophene soaps, compared with other bis-phenols as germicidal soaps:

TABLE 26

Conc. material in coconut oil soap	Results*			
	G-11	G-5-S	G-5	G-4
1-1000 in 5% soap	-0, -40 (+20, +85)	+45, +360 (+600)	+250, +400 (+450)	+350 (+450)
1-2500 2%	±150 (+250)	+250 (+750)	+650 (+750)	+850 (+2000)
1-5000 1%	+150, +350 (+750)	+900 (1500)	+800 (+950)	+1800 (+2500)
0 5%	around 1-4000			
0 1%	" 1-7000			

\* Numbers represent colony counts using F.D.A. phenol-coefficient procedure with Cade-Halvorson plate-count modification. Figures in parentheses represent data obtained when serum was used in the agar as an antidote (2 per cent). *M. pyogenes* var. *aureus* was the test organism. 37° C/5 minutes were the respective temperature and contact time employed.

Data like these, using gram-negative organisms instead of gram-positive ones are of no value for correlative purposes, for soaps *per se* are germicidal to a varying degree to gram-negative bacteria. Thus any result obtained would be that of the soap plus the medication and so one could not tell therefrom what part was due to which ingredient. In many cases the soaps *per se* are sufficiently germicidal for the purpose at hand and need no added ingredient when gram-negative organisms are the only ones to be considered. This is especially true of coconut oil soap or mixtures with a relatively high percentage of the latter. A few data to demonstrate these facts follow. This is really a separate subject—"Soaps as Germicides"—on which there are a legion of articles in the scientific and trade literature, and to which reference is made for additional information.

*Bacteriostatic Dilutions.*—In many practical applications where these bis-phenols (*e.g.*, hexachlorophene and some of its closely related congeners) have been found to serve a useful purpose, it is their bacteriostatic properties basically which produce the desired results. Therefore the fact that the bis-phenols may be considered as "slow-acting" germicides (as compared to iodine or chlorine

compounds, or even some of the newer type quaternaries) does not detract from their practical usefulness. This is true, for example, in such applications as for skin degerming, "athlete's foot" prophylaxis and treatment, prevention of diaper-rash, preventing skin infections due to pus-forming organisms, and a variety of other therapeutic applications.

TABLE 27.—GERMICIDAL DATA: SOAPS VS. *S. TYPHOSA*

<i>Soap tested</i>	<i>Dilution</i>	<i>Average result after 5 minutes/37° C contact</i>
Coconut oil soap	5%	—0
	2%	—0
	1%	—0
	0.5%	+550
	0.2%	+2500
Sodium oleate	5%	+200
	1%	+2500
	0.5%	+5000
Bar soap (about 20% coconut oil, the rest olive oil and tallow base)	5%	+0
	1%	+250
	0.5%	+2000
Ivory bar soap	5%	—0
" " "	2%	+2500
Lux " "	5%	—0
" " "	2%	+2500
Sodium oleate 100% + coconut oil soap 0%	5%	+8000
" 80% " 20%	5%	+350
" 60% " 40%	5%	—0
" 40% " 60%	5%	—0
<i>Medicated soaps (37° C/5 minutes)</i>		<i>S. typhosa data</i>
G-11 1-1000 in 5% coconut oil soap		—0
" 1-2500 2% "		—0 to —10
" 1-1000 5% sodium oleate		+300
" 1-2500 2% "		+2000
5% coconut oil soap		—0
2% "		—0
5% sodium oleate		+200
2% "		+2000

Data in the literature on the relative bacteriostatic potencies of the bis-phenols (G-4; G-5; G-5-S; G-11) vary over a rather wide range, but all investigators more or less agree that these compounds rate very high in this regard. Price and Bonnett (1948) report: "In dilutions as high as 1 to 5,000,000 or even 1 to 8,000,000 in agar media, G-11 causes complete or nearly complete inhibition of *M. pyogenes* var. *aureus*. This bacteriostatic effect disappears when the dilutions are in the neighborhood of 1 to 20,000,000. *E. coli* grows with only slight reduction of counts in 1 to 1,000,000 G-11 media." "1 to 4,000,000 (G-5) in nutrient agar culture media causes complete or nearly complete inhibition of *M. pyogenes* var. *aureus*.



Less than 10,000,000 has little or no bacteriostatic effect.—Mixed skin organisms, mostly *M. pyogenes* var. *albus*, appear to be slightly less susceptible to the bacteriostatic effect of G-5. *E. coli* is definitely more resistant.”

Seastone (1947), using a heavier inoculum than did Price, found three strains of normal skin staphylococci, tested in broth, were completely inhibited by G-11 at a dilution of 1 to 1,000,000, with partial inhibition at 1 to 10,000,000. Twenty strains were tested on agar at 1 to 1,000,000 dilution, 2 strains showed only partial inhibition. At 1 to 10,000,000 all 20 strains grew as well as on the control plates. Three gram-negative organisms (coliforms), which were tested in the same manner, grew in concentrations of G-11 10 to 20 times greater.

Maglio (1952*b*) states finding the bacteriostatic level of G-11 to be approximately 1 to 3,500,000; and for G-4, 1 to 750,000.

Shumard *et al.* (1953), and Hunter *et al.* (1953) give the following data as to the bacteriostatic activities of G-5-S (Actamer), based upon a 48-hour contact period on the organisms listed:

TABLE 28

*Dilution of actamer in parts per million  
producing complete inhibition*

<i>Test organism</i>	1000	100	10	1 0	0 1	<i>Control</i>
<i>M. pyogenes</i> var. <i>aureus</i>	—	—	—	—	+	+
<i>S. typhosa</i>	—	+	+	+	+	+
<i>E. coli</i>	+	+	+	+	+	+
<i>B. subtilis</i>	—	—	—	—	+	+
<i>Lactobacillus</i>	—	—	+	+	+	+
<i>Aerobacter aerogenes</i>	—	—	+	+	+	+
<i>Proteus vulgaris</i>	—	—	+	+	+	+
<i>Pseudomonas aeruginosa</i>	+	+	+	+	+	+
<i>Streptococcus faecalis</i>	—	—	—	+	+	+

TABLE 29

*Bacteriostatic end-point  
(M. pyogenes* var. *aureus)*

<i>Compound</i>	
G-4	1-500,000 to 1-750,000
G-5	1-2,500,000
G-11	“
G-5-S	“
G-11-S	“
3,3'-methylenebis(2,4,6-trichlorophenol)	1-500,000
4,4'-methylenebis(2,6-dibromophenol)	1-250,000

*E. coli*

G-4	Around 1-25,000
G-5	“ 1-10,000
G-11	Between 1-10,000 and 1-50,000
G-5-S	Around 1-10,000

Zone of inhibition data (F.D.A. method) also show bacteriostatic potencies, but the results do not necessarily correlate due to the fact that other properties (*e.g.*, diffusion rates through agar) enter into the situation producing the zone size. The following table shows relative zone-producing abilities of certain bis-phenols.

TABLE 30

Material tested	Dilution	Zone size in mm ( <i>M. pyogenes</i> var. <i>aureus</i> )	
G-4 in water*	1 1000: 1 10,000	5 0	2 0
G-5 "	" "	5 0	2 5
G-5-S "	" "	5 0	3 0
G-11 "	" "	5 0	3 0
G-4 in soap**	1 1,000 5% soap: 1 10,000 0 5%	2 5	0 0
G-5 "	" "	4 0	2 0
G-5-S "	" "	4 0	2 0
G-11 "	" "	4 0	2 0

\* Solubilized by alcoholic N 2 KOH(1 1 mol.)

\*\* Coconut oil soap. Results using other soaps such as Lux, Ivory, Dial blank soap, and sodium oleate parallel these quite closely.

A study of these data shows the bis-phenols to possess extraordinarily high bacteriostatic powers, but relatively low germicidal properties, based upon rapid action (up to 15 minutes contact time—the limit used for phenol-coefficient tests). However, to say or assume that these bis-phenols are poor germicides is incorrect, for if the contact time is increased, as would be the actual condition when they are used compounded into creams, lotions, etc., for application to the body or skin, then they produce good practical germ-killing action. The following tabulation shows these facts (data from Sindar laboratories):

TABLE 31

		37°C <i>M. pyogenes</i> var. <i>aureus</i> data		
Substance	Dilution	5 min	30 min	5 hrs
G-11	1 1000 in 5% soap (coconut)	-50(+240)	-0(-2)	-0(-0)
G-11	1 5000 1%	+240(+300)	+2(+10)	-0(-0)
G-5-S	1 1000 5%	+350(+360)	+6(+8)	-0(-0)
G-5-S	1 5000 1%	+900(+1000)	+240(+420)	-0(-0)

Figures in parentheses are data as obtained when using serum in the agar as an antidote.

**Antifungal Activities.**—Many of the bis-phenols show high antifungal properties, both fungicidal and fungistatic, against many of the wide variety of multicellular organisms classified as fungi. Their activity is in no way correlative to their germicidal powers.

Compounds highly effective against bacteria might have equally good or relatively poorer activity against certain fungi. Furthermore some bis-phenols are extremely potent against one type of fungus (*e.g.*, cellulose decomposers) but may require many times the effective concentration for preventing the growth of these to bring about a similar growth-prevention or killing of protein destroying organisms. Also, compounds effective against these cellulose and protein destroyers may or may not be potent against those pathogenic types of fungi known as dermatophytes which cause primarily skin diseases in man and animals. Each must be tested for its specific purposes and cross-correlations because the assumption that a product good for one type of fungus will be good for another cannot be made with any degree of accuracy.

A few tables of data giving general type information along these lines follow, showing individual activities, as well as some correlations of chemical structure to activity.

Pfleger, Schraufstatter, Gehringer, and Sciuk (1949) published the results of a very extensive piece of research on the general subject of chemotherapy of fungus-infections in which they present data on the bacteriostatic and fungistatic dilutions for some 45 bis-phenols with sulfur linkages. *M. pyogenes* var. *aureus* and *S. paratyphosa* were the bacteria used, with *Tr. gypsum* and *Tor. minor* the fungi tested. A condensation from their data follows:

TABLE 32

Compound	Growth-inhibition concentrations	
	<i>Tr. gypsum</i>	<i>M. pyogenes</i> var. <i>aureus</i>
2,2'-thiobis-phenol	1 320,000	1 30,000
4,4'-thiobis-phenol	1 40,000	1 10,000
2,2'-thiobis(4-chlorophenol)	1 2,560,000	1 640,000
2,2'-thiobis(4,6-dichlorophenol)	1 10,000,000	1 1,280,000
2,2'-thiobis(4-chlorophenylacetate)	1 5,120,000	1 640,000
2,2'-thiobis(4,6-dinitrophenol)	1 20,000	< 1 20,000
2,2'-thiobis(4-chloro-5-cresol)	1 1,280,000	1 1,280,000
2,2'-thiobis(4-chloro-3,5-xyleneol)	1 20,000	< 1 40,000

See original paper for many other similar type compounds, and for data on the other organisms used.

Schraufstatter, Richter, and Ditscheid (1949) published another paper on the same general subject, with special reference to their compound "D-25" (same as G-4-S), 2,2'-thiobis(4-chlorophenol). Several tables of data are given showing correlative results of limiting growth concentrations of various chemicals used for



fungicidal purposes. A condensation of their data, to show in brief the general relationships, follows:

TABLE 33

<i>Compound</i>	<i>Growth-inhibition concentrations (Tr. gypseum)</i>
Resorcinol	1-1000
Hydroquinone	1-1000
Salicylic acid	1-4000
Hexylresorcinol	1-160,000
Undecylenic acid	1-160,000
Salicylanilide	1-120,000
8-Hydroxyquinoline	1-640,000
Gentian Violet	1-640,000
"D-25"	1-2,560,000
<i>Test organism</i>	<i>Effective growth-prevention conc. Cpd. D-25</i>
Tr. gypseum	1-2,560,000
Tr. lacticolor	1-640,000
Ep. interdigitale	1-2,560,000
M. audouini	1-2,560,000

Marsh and Butler (1946) report a series of biological tests run to determine the fungicidal potencies as mildew-preventives on cotton fabric of a group of bis-phenols and related compounds. The correlated effects of various substituent groups were studied. The authors report "no compound was found to be more fungicidal per unit weight than 2,2'-methylenebis(4-chlorophenol)." Other compounds tested included congeners wherein the  $-CH_2-$  linkage was replaced by an  $-S-$ , a  $-CO-$ , and a carbon to carbon bond.

Horsfall *et al.* (1951) present a very extensive survey showing the toxicity of a large number of diphenyl compounds, including bis-phenols, to certain fungi. *Stemphylium sarcinaeforme* and *Sclerotinia fructicola* were the test organisms used. The relative effects of various types of bridges connecting the two phenyl rings, and of different substituent groupings on the rings themselves are noted and correlations discussed.

Shirk and Corey (1952) report on a study correlating the efficacies of various configurations of monophenols, comparing their activity with that of G-4.

Watkins and Klemme (1948) published a paper on their findings showing the effect of varying the amount of dextrose in the agar media on the weight of mycelium produced by various fungi growing in the presence of 2,2'-methylenebis(4-chlorophenol).

Shumard *et al.* (1953) and Hunter *et al.* (1952, 1953) present the following table of data showing the fungistatic potency of Actamer against several of the common organisms, pathogenic and non-pathogens.

TABLE 34  
*Fungistatic activity of Actamer in ppm*

Organism	1000	100	10	1 0	0 1	Control
Tr. gypseum	—	—	—	+	+	+
Ep. inguinale	—	—	—	+	+	+
Ch. globosum	—	—	+	+	+	+
P. notatum	—	—	+	+	+	+
P. citreum	+	+	+	+	+	+
A. niger	+	+	+	+	+	+

## PRACTICAL APPLICATIONS

*Skin Degerming; Skin Antiseptics.*—It is in the field of skin antisepsis (sometimes misnamed as skin disinfection) and of skin degerming that the bis-phenols of the hexachlorophene type have come to the fore as exceptional agents.

Work on the effectiveness of G-11 on reducing the bacterial flora of the skin was started by Cade and Gump in 1942. This was followed by an extensive study carried out by Traub, Newhall, and Fuller (1944, 1945). The results of their findings brought to light, for the first time, the valuable effect of the continuous use of G-11 compared to the much poorer results which one-wash applications showed. They report finding "the evidence indicates that the regular use of toilet soap containing compound G-11 in a concentration of 2 per cent has such an effect on the bacterial flora of the human skin that the so-called resident bacteria are decidedly and permanently (as long as this soap is used) reduced in number."

Udinsky (1945), using a simplified swab technique, found similar results as far as the effect of continued use of G-11 soap had on reducing the normal skin flora count.

Soon after these findings appeared, several other medical men extended this work into other applications of their own interests, each showing the successful application of hexachlorophene, especially for skin degerming purposes, or to supplant or supplement the standard "surgical scrub-up" procedure for physicians, nurses, etc. Some have used the hexachlorophene in soap solutions, others have worked with pHiso<sup>(R)</sup>hex, G-11 being incorporated in a soapless detergent cream base pHiso<sup>(R)</sup>derm, or with combinations of G-11 and other detergents. G-11 in organic solvents was also employed.

Seastone (1947), using 1 per cent G-11 on the soap-weight basis in liquid soap states that he found it to accomplish a bacterial reduction of about 1000 fold from normal skin count, as compared with the conventional scrub followed by germicidal rinses, which reduces the number about ten-fold.

Fahlberg, Swan, and Seastone (1948) found that the presence of G-11 in the skin can be demonstrated up to 2 days after 3 consecutive daily 6-minute applications of a 1 per cent solution in liquid soap of 20 per cent strength. A unique method for determining the retention of G-11 in the skin is described.

Seastone and Erickson (1949) compared the efficiencies of liquid *vs* solid soaps containing G-11, and state that in a liquid soap, G-11 was more effective for surgical scrub-up than when in a solid soap, with the necessary contact time being reduced from 6 to 1 minute.

Best, Coe, McMurtrey, and Henn (1950), using 2 per cent G-11 in bar soap (Dial), found that a 3-minute period was sufficient for the surgical scrub-up, with the total scrub time lengthened to 5 minutes if the soap is not used daily by the surgeon.

Best, Coe, and McMurtrey (1951) tested the efficiency of 2 per cent G-11 in bar soap, and 1 per cent in a 20 per cent liquid soap on the tissue of wounds and on burned surfaces. They reported finding no untoward tissue reactions.

Blank and Coolidge (1950) determined the variations in bacterial flora counts of the skin weekly, for several weeks, when G-11 in soap and in pHisoderm was used for general personal hygiene purposes. They state that for preoperative preparation, a single wash with hexachlorophene was found to be as effective, but not more so, as the routine 10-minute scrub-up.

Blank, *et al.* (1950) report finding that rapid degerming of the skin is not accomplished by scrubbing with a 2 per cent G-11 bar soap or with pHisoderm containing 3 per cent G-11 followed by a 1-minute dip in 0.1 per cent Zephiran<sup>(R)</sup>, but it is obtained by a 2-minute scrub with ordinary soap and brush, after which the hands and arms are kept wet for 2 minutes by continuous or repeated immersion in a solution of 0.1 per cent G-11, and of 0.5 per cent cetyl alcohol, in 70 per cent (by volume) isopropyl alcohol.

Vestal Laboratories, Inc. (1949) in special scientific bulletins present excellent and extensive data showing the effectiveness of G-11 in liquid soap as a skin degerming agent.

For other information and papers on this general subject the following additional publications are listed:

Allers *et al.* (1950); Artz *et al.* (1951); Bowers (1949); Brady (1952); Cade (1950, 1952); Chisholm *et al.* (1950); Clark



*et al.* (1947) ; Dull *et al.* (1950) ; Freeman and Young (1949, 1950) ; Fuller *et al.* (1948) ; Gill (1950) ; Horsey (1949*b*) ; Hufnagel *et al.* (1948) ; Kraissl (1950) ; Lord *et al.* (1953) ; McDonald *et al.* (1949) ; Nungester *et al.* (1949) ; Painter (1950) ; Price and Bonnett (1948) ; Price (1951*a, b*) ; Reid *et al.* (1950) ; Shay (1951) ; Sindar (1950) ; Spoor and Traub (1951) ; Thirlby and Nesbit (1949) ; and Traub (1949).

Other pertinent references appear in many of the above mentioned papers, especially those by Gump (1945), Maglio (1948, 1952*a*), Kraissl (1950), and in a complete bibliography published as Bulletin H-1, May 1952, by Sindar Corporation.

A variety of test procedures has been described in the literature for determining the efficacy of the bis-phenols or other antiseptics for skin degerming purposes. Different methods (direct and indirect) have been used as criterions of relative efficiencies of the compounds being tested. These include, in brief, such processes as (1) the removal of bacteria for counting by swabbing skin surfaces (Udinsky, 1945) ; (2) by excising skin pieces (Kraissl, 1950), (Best *et al.*, 1951), (Artz *et al.*, 1951) ; (3) by placing agar discs on skin areas or skin areas on agar surfaces (Best *et al.*, 1950), (Painter, 1950), (Shay, 1951), and Novak (1939) ; (4) by using small glass cylinders placed on the skin surfaces and filled with saline or other solutions, followed by swabbing the skin area inside the cylinder and plating an aliquot portion of the liquid (Cornbleet, 1932) (Pillsbury and Nichols, 1946) and (Killian, 1950) ; (5) by *in vivo* infection-prevention methods (Bowers, 1949), (Nungester *et al.*, 1949), (Thirlby and Nesbit, 1949), and (Freeman and Young, 1950) ; (6) by analyzing "glove-juice" for bacterial counts (Bowers, 1950), (Reid *et al.*, 1950) ; (7) by washing the total skin area chosen (hands and arms to elbow) and plating the wash-water.

This latter method is now employed by most workers in this field, being a modification in one form or another of the original Price Multiple Basin technique (Price, 1938*a, b*), and expanded to a more practical use by Pohle and Stuart (1940, 1941), applied by Traub and his co-workers (1944, 1945), and still further modified for skin-degerming efficiency tests by Cade (1950, 1951, 1952), and by Gump and Cade (1952) to fit the practical needs of comparative evaluations of bis-phenols as degerming agents.

*Skin Deodorants.*—Another practical application, making use of the germicidal and bacteriostatic properties of bis-phenols, is for body deodorant purposes. Experimental evidence has been presented to indicate that perspiration as it is excreted on the skin of many, if not most, normal humans is low in bacterial count, and possesses but little or no objectional odor (Barail 1946, 1947) (Kil-

lian and Panzerella 1947). Furthermore, when this perspiration is kept under sterile conditions no additional off-odors are formed; but when it is allowed to stand "as is," growth of bacteria occurs and bad odors are produced. On the basis of these quite well-established facts, it has been shown by Killian (1952) that if a person with normal perspiration should use a material which would prevent bacteriological growth and decomposition to go on in the perspiration while on the skin surface, there should be a marked decrease in odor formation. Where the perspiration odor is basic for the individual, due to some personal physiological characteristic, and appears in the perspiration as it is secreted (a normal condition for some people), then a bacterial growth-prevention situation, set up by a chemical such as hexachlorophene, would not bring about a complete neutralization of the odor, although there would be a distinct reduction over the extent of the odor that would occur should the hexachlorophene (or its equivalent in bacterial growth prevention) not be used. Practical applications of these reasonable assumptions tend to show that in general they are correct, but more actual scientific data are needed to prove these facts conclusively.

*Disinfectants and Detergent Sanitizers.*—Due primarily to the fact that bis-phenols as manufactured today cost more than certain mono-phenols, they have not appeared competitively in the field of disinfectants, although they possess good germicidal powers when compounded for disinfectant purposes. Some of the bis-phenols have actually found use in industrial disinfectants, but up to the present time this use is limited.

Dissolved in soap solutions, in a manner similar to that as employed for other phenolics (*e.g.*, cresols, phenyl- and benzyl-phenols, etc.) some of the bis-phenols (*e.g.*, G-4 and G-4-S) show comparatively equivalent germ killing powers. So where other factors such as absence of objectionable odors or decreased irritation properties are sufficient to over balance the cost differences these bis-phenols can be and are being used as the base for disinfectants.

Attention is called here to the fact that these disinfectant solutions, although having soap as their base, are not classifiable as germicidal soaps. Compounds belonging to the latter class have the soap present in predominating amounts (*e.g.*, 50 to 1 to 20 to 1 soap-phenol ratio), whereas to produce good disinfectants this ratio must be close to 1 to 1. It has been shown (Cade and Halverson, 1934; Cade, 1935) that the ratio of phenol to soap played a very important role in determining the efficacy of these mixtures, and that this fact applied not only to phenols and cresols but also to the phenyl- and benzyl-phenols, which were at that time just coming into practical use available for disinfectant compounding purposes.

Later this same phenomenon was found, as would be expected, to apply to the bis-phenols.

As for the application of bis-phenols in the field of sanitization, little scientific information is available at the present time. The same general comparisons, as mentioned above for disinfectants, should follow however, since these two biological processes differ only in a matter of degree. Disinfection requires 100 per cent killing of the test organisms, whereas sanitizing usually requires only the killing of a sufficient number of the organisms present to render the product safe from a public-health standpoint.

Finally, attention should be called to certain facts that pertain to the compounding of mixtures containing bis-phenols to be used for detergent (germicidal or sanitizing) purposes. Being anionic substances, naturally, the bis-phenols under most practical application conditions are incompatible with quaternary ammonium compounds, which are also used quite extensively for detergent-sanitizer purposes. Likewise, as in the case of all phenols (including the phenyl- and benzyl-phenols), the presence of alkalis decreases the germicidal potencies of some of the bis-phenols due to changes in ionization.

Alkali builders, other type detergents (anionic and nonionic), and sequestering agents such as are usually added to mixtures used for detergent purposes, all play a role in determining the net efficiency of the mixture. Some materials may exert a useful synergistic action, while others may produce an undesirable neutralizing or antagonistic effect. In some mixtures, naturally, it would be possible for both types of action to be present and neutralize each other. Suffice it to say here, the experimenter in this field should recognize these possibilities when attempting to compound satisfactory mixtures for germicidal-detergent or detergent-sanitizer products.

Spaulding (1946), found that adding 0.5 per cent G-11 to a formaldehyde sterilizing solution enhances the efficacy of the latter such that the killing time of spores treated with it can be reduced from 18 to 3 hours, while that for the corresponding vegetative forms is reduced from 30 to 20 minutes.

## APPLICATIONS IN INDUSTRY

*Mildew- and Rot-Proofing.*—Although the original literature on the use of bis-phenols mentions activity against fungi, it was primarily as moth-proofing agents that the compounds presented were found to be especially effective. No special mention was made as to the activity of any particular bis-phenol against any specific type or species of fungus.



Actually, it was not until about a decade and a half ago that the practical excellent mildew-proofing properties of some of these bis-phenols were discovered. This applies especially to "dichlorophene," known in the trade as G-4<sup>(R)</sup> or Preventol<sup>(R)</sup> G.D. The pioneering work on this application was carried out in the Sindar laboratories (1940), followed cooperatively and independently by works of others, principally Marsh, Greathouse, and their co-workers (1945, 1946); as well as in several government laboratories such as the Bureau of Standards, Chemical Corps, Ordnance Department, Quartermaster Depots, Corps of Army Engineers, Signal Corps, etc.

As the result of all of this work by government agencies on deterioration prevention, G-4 was found to be among the best mildew- and rot-proofing agents as used by the armed services. Many government specifications now require or recommend its use. It is highly effective against cellulose decomposing organisms, such as *Chaetomium globosum* and *Metarrizium* sp., in strengths around 0.2 to 0.5 per cent on fabric weight; while against the protein decomposers such as *Aspergillus* and *Penicillium*, etc., it is also active, but to a lesser degree, usually requiring 1 to 3 per cent on the basis of material weight for full protection. For materials which must meet the soil burial test requirements intermediate amounts are generally satisfactory.

A considerable volume of literature is available on the use of G-4 for mildew- and rot-proofing purposes. Reference is also made to "Microbial Decomposition of Cellulose" by Siu (1951) for a considerable discussion on this subject. A few of the more important papers available on this specific subject are those by Abrams (1948); Bayley (1947); Bayley and Weatherburn (1947); Barker and Marsh (1949); Berk (1947, 1948 *a b*); Bertolet (1943); Block (1946, 1948); Goodavage (1943); Grupp (1948); Horsey (1949*b*); Marsh (1947); Marsh and Butler (1946); Marsh *et al.* (1945); Watkins and Klemme (1948); and Sindar's Technical Bulletin 49-2 (July 1949)

*Antifungal Applications Against Human, Animal, and Plant Diseases.*—Little information in the form of scientific reports is available on the use of bis-phenols as antidermatophytes, although it is known that some of these products have found rather wide, and apparently successful usage in the field. Udinsky (1945) reports good results with a G-4 ointment especially in patients who resisted other forms of treatment. Sweatman (1947), referring to application to treatment of certain animal infections states that G-4 ointment is a superior preparation, being efficient in "arresting the spread and allowing the healing of ringworm lesions." Somewhat

similar results are reported by Brook (1949). Richter (1950) found "D-25" (G-4-S) of value in the treatment of various mycoses on topical and internal application, stating that therapeutically effective blood levels were obtained by oral and intramuscular administration. Richter considers D-25 to be the first specific internally active fungistat in the chemotherapy of mycoses. Goldsworthy (1949) found G-4 to be one of the ten most promising plant fungicides for peach trees, of 506 compounds tested.

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## 13

# ESSENTIAL OILS

THE practical application of the essential oils for the control of, or for the prevention of, deleterious effects due to micro-organisms on both animate and on inanimate objects dates back to antiquity. Such compounds, or substances containing them and classifiable as essential oils, find usage today in the field of antiseptics, germicides, or fungicides. Some find application as preservatives in the food industry, as well as in the field of cosmetics, pharmaceuticals, and in the toilet-goods industry. Others are used for the prevention of deterioration due to both bacterial and fungal growth on textiles, paint surfaces, leathergoods, etc. Application of certain essential oils is also employed for therapeutic purposes in both bacterial or fungal diseases of man and animals. Essential oils do not find very wide use today as disinfectants and sanitizers because many other compounds are available for these purposes, which are cheaper and are active in higher dilutions.

### DEFINITIONS AND CLASSIFICATIONS

It is difficult, if not impossible, to establish an all-inclusive definition for an essential oil, sometimes also known as a volatile oil, or an ethereal oil. A basic definition might be considered to be: an odoriferous oily substance obtained from natural sources, usually plants, distillable with steam. This definition will undoubtedly include the majority of those compounds commonly included in the group, and at the same time exclude the fixed oils and those materials with odor which are not distillable with steam. However, there are certain other substances which probably belong in the essential oil classification but which do not meet all these requirements. Some of these exceptions might partially decompose during steam distillation, but can be obtained from their natural habitat by solvent extraction or by pressing, etc. The essential oils, thus defined, may be classified in a variety of ways, such as botanical properties,

or on the basis of application, or according to the principal constituent which they contain, such as hydrocarbons, phenols, alcohols, aldehydes, ketones, acids, esters, or as antiseptics, germicides, disinfectants, preservatives, bacteriostats, sanitizing agents, etc.

## CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY

All essential oils, as obtained from nature, are a mixture of a variety of substances. They are not individual chemicals representable by any specific structure or formula. The oils are a mixture or blend of various chemicals, with usually one of the component parts predominating both as to amount and as to biological potency. Some are basically hydrocarbons, for example, the terpene group; others have an alcohol, aldehyde, or ketone type product as their principal constituent; still others predominate with an ester or ether compound; while, in others, acids and phenolic substances may be found as the basic materials. These oils may possess widely different degrees of activity as far as their effects on micro-organisms are concerned.

The activity of any one oil is not necessarily due to just one component part, even though it may predominate in the pure material, but is due to the net result of the effects of the several components. In some cases these effects may be additive, while in others they may be synergistic, or even the opposite, that is, antagonistic to each other. In other words, these oils produce a summation effect, with the respective isolates producing their own activities, combining to their respective net results.

The relative efficiencies of these essential oils are not necessarily in proportion to the type of their major component ingredient, nor to the percentage present in the basic oil. Therefore, relative effectiveness cannot always be determined by chemical analysis. Furthermore, the germicidal efficiencies may not necessarily parallel their respective antiseptic or preserving properties. Similarly, their bactericidal and/or bacteriostatic properties may not parallel their corresponding fungicidal and/or fungistatic activities. There are a wide variety of factors which determine the net result in each individual case. In addition, there are also other factors such as nature and type of micro-organism involved, and the environment in which the oil or its isolate is present. Certain generalities can be assumed but the real proof must be determined by experimental evidence. Many essential oils high in phenolic components may be more effective biologically than others. Terpenes and certain other hydrocarbon type oils are the least effective with aldehydes and ketone intermediates. Acids, as a rule, will be found



to be less active than phenols but more effective than their corresponding ester or ether derivatives, with their alkali salts in between. Preservative properties do not vary in the same degree as germicidal activity. Many compounds, oils and their isolates or synthetics of the latter, show good bacteriostatic powers and thus serve as good preservatives, but fail to effect good germicidal action. Also, activity in the vapor state differs in degree from that produced by the same material in liquid form.

Furthermore, a set of experimental data may be accurate in itself, but the conditions under which it was obtained may differ so much from that which produced other data, with which it is being compared, no accurate comparison between them can be made. This may be due to the fact that some may have used pure oils while others employed crude extracts, or natural products with and without essences added, or even mixtures of unknown composition. For these reasons, the literature on the germicidal and antiseptic properties of essential oils is often confusing and of little value for comparative purposes.

Since most of the essential oils have such low solubility in water, they cannot be tested without the use of some other solvent or emulsifying agent, such as alcohol, aqueous emulsifying agents, soap mixtures, etc. Increase in solubility, together with decrease in particle size due to the emulsifying agents used, results in differences in the activity of the oils being tested.

Likewise, formulations highly active against one species of micro-organism may be far less effective against another. This also applies to various strains of the same test organism. Thus when comparisons are being made of the effectiveness, or possible usefulness, of a group of essential oils, consideration must be given to the test organism used in the evaluation, and comparisons must be based upon tests against the same organism. The activity of one substance against gram-positive bacteria often differs markedly from that against gram-negatives, and the activity of the same essential oil material against fungi may be markedly different from that against bacteria.

## HISTORICAL

The recognition of essential oils, especially in their crude state such as spices, oleoresins, etc., and their use on or for humans, dates back to antiquity. In fact, information obtained from early literature and prehistoric relics indicates, quite conclusively, that these oils were known and used several thousand years prior to Biblical days, for adorning, religious, odorant, as well as for preservative purposes.

The discovery of the relationship which exists between micro-organisms and disease, which occurred around the beginning of the 19th century, brought forth a more extensive use of the essential oils and their isolates, etc. for the purpose of controlling microbial growth and/or of the effects of the latter on the host or on the environment in which it occurs (i.e. as a preservative against decomposition due to fermentation and/or putrefaction).

At first only the crude natural materials were employed, such as spices, bark, herbs, roots, flowers, etc. Later, after the process of distilling was discovered and perfected, the respective constituent oils were obtained from these crude sources. Still later when the means for separating these oils into their component parts were developed and the individual chemicals obtained, and with further advancement in both the art and science of chemistry, accurate knowledge was obtained as to the chemical structure of these various essential oils, the preparation of the same substances synthetically naturally followed.

Among the earliest to recognize and describe antimicrobial activities of essential oils were Schonbein in 1840, de la Croix (1881), Miller (1892), Harlan (1888), Freudenreich (1889), and Mawhinney (1900).

Chamberlain (1887) used the Koch thread method and suspended bacteria in sealed tubes over the respective oils. Oil of cinnamon was the only essential oil of those tested to accomplish the desired result. It required 4 days to kill anthrax spores, whereas the vegetative cells were killed in 18 hours by oil of vespetro, in 40 hours by oil of angelica, and in 65 hours by oil of cinnamon.

Cadeac and Meunier (1889) also tested the activity of some essential oils against *Bacillus anthracis*. They found oils of cinnamon, clove, thyme and geranium effective within 12, 15, 35, and 50 minutes respectively. They also exposed *Salmonella typhosa* to the essential oils and found oil of cinnamon to kill the organisms in 12 minutes, clove in 25, thyme in 35, and geranium in 50 minutes respectively.

Kobert (1906) determined the inhibitory activity of certain essential oils by adding milk and powdered sulphur to the oils, inoculating the mixture with bacterial culture, incubating for 24 hours and determining the end-point of inhibition by observing the presence or absence of  $H_2S$  formation.

Martindale (1910, 1911) presented results of the first really extensive research on the bactericidal properties of essential oils, using quantitative test procedures. He employed a modified Lancet method, using both aqueous and soap solutions of the oils. Phenol-coefficients of 50 or more different oils and/or their isolates were

obtained, using *Escherichia coli* as the test organism. Some of these results are given in Table 35.

TABLE 35

<i>Essential Oil or Constituent Tested</i>	<i>Phenol-coefficient at 2 minutes and 30 minutes</i>		<i>Average Phenol-coefficient</i>
Origanum (aq.)	28	23.5	25.76
Thymol (soap)	30	20.54	25.29
Carvacrol (soap)	25	17.6	21.32
Thymol (aq.)	20	18.8	19.4
Geraniol (soap)	14	10.58	12.29
Cinnamon leaf (soap)	10.5	8.82	9.66
Clove (soap)	11	6.76	8.88
Cinnamic aldehyde (soap)	9	7.0	8.0
Citronella (soap)	8	8.2	8.1
Cinnamon (aq.)	8.3	5.88	7.1
Rosemary (aq.)	6	5.88	5.9
Cassia (soap)	6	4.7	5.35
Wintergreen (soap)	4	5.29	4.64
Eucalyptus (soap)	4	4.7	4.35
Lavender (soap)	4	5.88	4.94
Lemon (soap)	2	5.88	3.94
Almond (soap)	4	3.53	3.76
Eucalyptol (soap)	4	3.53	3.76
Sandalwood (soap)	1	2.35	1.67

Hoffman and Evans (1911) studied the preservative action of cinnamon, cloves, mustard, allspice, nutmeg, ginger, black pepper, cayenne pepper, etc., and found the first three most effective, the last three least.

Cochran and Perkins (1914, *a, b*) tested the efficiency of certain essential oils, and spices containing them, as preservatives against the growth of yeasts in sugar and starch solutions, as well as in certain foods. Among the oils found useful were oils of birch, cassia, star-anise, sassafras, ginger, lemon, orange, wintergreen, spearmint, peppermint, and citronella.

Bachmann (1916, 1918, 1923) in a comprehensive series of tests used ground spices, together with alcohol extracts, the essential oils themselves, and their active principles. The test organisms used included *Rhizopus*, *Penicillium*, and *Aspergillus*; and the bacteria *Serratia marcescens*, *Escherichia coli*, and *Bacillus subtilis*. The author concluded that spices used in amounts as employed normally in the kitchen for ordinary foods were insufficient as preservatives. When used in larger amounts, cinnamon, clove and allspice retarded mold growth.

Cavel (1918) tested some 45 different essences dissolved in solvents, incorporated in broth and inoculated with sewage organ-



isms. His data, the dilution per 1000 ml at which the respective oils no longer showed inhibition action, are given in Table 36.

TABLE 36

<i>Oil Tested</i>	<i>Inhibitory Dilution</i>	<i>Oil Tested</i>	<i>Inhibitory Dilution</i>
Thyme . . . . .	0.7	Anise . . . . .	4.2
Origanum . . . . .	1.0	Mustard . . . . .	4.2
Cassia . . . . .	1.7	Rosemary . . . . .	4.3
Rose . . . . .	1.8	Lavender . . . . .	5.0
Clove . . . . .	2.0	Juniper . . . . .	6.0
Eucalyptus . . . . .	2.25	Garlic . . . . .	6.5
Peppermint . . . . .	2.5	Lemon . . . . .	7.0
Geranium . . . . .	2.7	Sassafras . . . . .	7.5
Wintergreen . . . . .	3.2	Heliotrope . . . . .	8.0
Spike . . . . .	3.5	Turpentine . . . . .	8.4
Star anise . . . . .	3.7	Violet . . . . .	9.0
Ceylon cinnamon . . . . .	4.0	Camphor . . . . .	10.0
Birch . . . . .	4.8	Patchouli . . . . .	15.0

Gatti and Cayola (1922*a, b*; 1923, 1924) listed the various essential oils tested according to the following classification, based upon the degree of activity as noted, as shown in Table 37.

TABLE 37

<i>Highly Active</i>	<i>Active</i>	<i>Moderately Active</i>	<i>Inactive</i>
Clove	Eucalyptus	Bergamot	Neroli
Wintergreen	Lavender	Rose	Geranium
Sassafras	Sage	Patchouli	Vetiver
Sandalwood	Violet	Verbena	Jasmin
Thyme			Opopanax
Cinnamon			Origanum
Camphor			Lemongrass

These lists are based upon the action of these oils against several organisms such as *Micrococcus pyogenes* var. *aureus*, *Streptococcus pyogenes*, *Penicillium glaucum*, and *Aspergillus albus*.

Morel and Rochaix (1922, 1925, *a, b, c, d*; 1927, *a, b*) compared a series of essential oils in the liquid state with the same oils in the vapor state. The test organisms used were *S. typhosa*, *M. pyogenes* var. *aureus*, diphtheria bacillus and anthrax spores. The order of activity of these oils is given in Table 38.

These authors reported that oils of lavender, birch, mint, camphor, sassafras, savin, bay, and rosemary were effective for the treatment for affections of the scalp and that oil of gardenbalm,

mint, camomile, and cajeput possessed therapeutic value, having remarkable sedative action on the gastric mucous membrane. Oils

TABLE 38

<i>As a Vapor</i>	<i>(Order of Decreasing Activity)</i>	<i>In Fluid State</i>
Citron		Thyme
Thyme		Citron
Orange		Juniper
Bergamot		Menthol
Juniper		Gomenol
Cloves		Orange
Citronella		Citronella
Lavender		Clove
Gomenol		Lavendar
Menthol		Rosemary
Rosemary		Bergamot
Sandalwood		Eucalyptus
Eucalyptus		Sandalwood
Aniseed		Aniseed

of eucalyptus, pine, myrtle, myrrh, violet, basil, angelica, and thyme were effective, in this decreasing order, in diseases of the respiratory organs (Morel, *et al.*, 1928 *a, b, c*; 1937).

TABLE 39

<i>Oil Tested</i> (1 per cent aq. sol.)	<i>(8-hr. Contact)</i>	<i>Per Cent</i> <i>Efficiency</i>
Rosemary . . . . .		6.8
Turpentine . . . . .		10.0
Lemongrass . . . . .		10.0
Anise . . . . .		10.8
Lemon . . . . .		10.8
Pimento . . . . .		12.8
Orange . . . . .		12.8
Eucalyptus . . . . .		13.0
Sassafras . . . . .		18.5
Caraway . . . . .		18.5
Camphor . . . . .		20.0
Citronella . . . . .		20.0
Birch . . . . .		20.0
Clove . . . . .		25.0
Lavender . . . . .		26.0
Peppermint . . . . .		28.0
Bergamot . . . . .		29.6
Thyme . . . . .		30.8
Pennyroyal . . . . .		39.0
Wintergreen . . . . .		44.0
Cinnamon . . . . .		48.3

Harvey (1922, 1923, 1924, 1928) published considerable material covering his studies on the activity of essential oils as anti-

ferments, in which he determined the effect of the oils on the anti-hydrolyzing power of 1 per cent aqueous solutions of yeast-sugar mixtures. The results reported are shown in Table 39.

Bryant (1924 *a, b*) compared detergent values with antiseptic activity of various essential oils and noted a direct correlation between these two properties. He also noted that a large number

TABLE 40

<i>Essential Oil</i>	<i>Phenol-coefficient</i>	<i>Synthetic</i>	<i>Phenol-coefficient</i>
Almond	4.5	Acetophenone	4.2
Aniseed	3.5	Amyl alcohol	4.0
Bergamot	4.0	Anisic aldehyde	7.2
Caraway	5.4	Cinnamic alcohol	5.0
Cassia	6.0	Cinnamic aldehyde	8.8
Citronella	5.5	Citronellol	8.6
Cloves	8.5	Citronellal	12.4
Geranium	6.5	Citral	18.8
Lavender	4.4	Coumarin	3.2
Lemon	3.8	Eugenol	14.4
Neroli	5.5	Geraniol	11.5
Orange, sweet	2.2	Heliotropin	2.8
Otto rose	7.0	Isoeugenol	10.2
Otto orris	3.5	Linalool	7.0
Patchouli	1.8	Linalyl acetate	3.5
Petigrain	3.5	Methyl salicylate	5.8
Rosemary	5.2	Methyl benzoate	3.0
Sandalwood	1.2	Phenylethyl alcohol	9.0
Thyme	12.2	Safrol	10.8
Verbena	9.2	Isosafrol	12.6
Wintergreen	4.5	Terpineol	7.8
		Vanillin	5.4

of commercial perfumes possessed good antiseptic properties. A resumé of his results is given in Table 40.

In another series of tests Bryant determined the phenol coefficients of a variety of essential oils incorporated into bar soaps (1 per cent of soap weight) and found them to range between 0.1 to 0.5. *E. coli* was the test organism used in these and the above tests.

Dyche-Teague (1924) also presented considerable data on the bactericidal value of commercial perfumes, showing many of these to be active. The effect of aging on the germicidal potencies of perfumes, with and without synthetics incorporated therein, was also studied, showing the effect to be rather small. The culture used for these tests was obtained from nasal secretions and consisted primarily of *Micrococcus catarrhalis*.



Penfold and Grant (1922, 1923, *a*, *b*, 1924, 1925, 1926) conducted extensive studies on the germicidal values of various Australian essential oils, mostly varieties of eucalyptus, including also certain isolates from these oils, such as cymene, thymol, menthol, menthone, borneol, citronellol, camphor, safrol, isosafrol, etc. A condensation of some of their extensive data is given in Tables 41 to 46.

TABLE 41.—COMMERCIAL EUCALYPTUS OILS

<i>Crude Oil</i>	<i>Principle Active Constituent</i>	<i>Phenol Coefficient</i>
<i>E. australiana</i>	Cineol	5
<i>E. dives</i>	Piperitone	8
<i>E. radiata</i>	Piperitol	10
<i>E. citriodora</i>	Citronellal	8
<i>E. encorifolia</i>	Cineol	7.5

TABLE 42.—PURE CONSTITUENTS OF EUCALYPTUS OILS  
(FRESHLY PREPARED)

<i>Constituents</i>	<i>Nature</i>	<i>Phenol Coefficient</i>
Cymene	Hydrocarbon	8
Cineol	Oxide	3.5
Pinene	Terpene	1
Phellandrene	"	1
Limonene	"	1
Terpineol	Alcohol	7.5
Geraniol	"	21
Geranyl acetate	Ester	0
Citral	Aldehyde	19.5
Citronellal	"	13.3
Phellandral	"	9.25
Piperitone	Ketone	8

Myers and Thienes (1925) studied the activity of essential oils against certain types of yeasts. They report that thymol and oil of cinnamon are better than 1 per cent phenol for this purpose, with clove oil about equal to phenol in effectiveness, and menthol, peppermint, camphor, methyl salicylate, turpentine, eucalyptus, and lemon oil less effective than phenol.

Myers (1926, 1927) classified the oils tested as to their comparative fungicidal activity against a pathogenic yeast, and reported the number of minutes required to destroy the test culture. Thymol required less than 1 minute, carvacrol 1+ minute, mustard 10 minutes, cinnamon 30 minutes, clove 90 minutes, eugenol, turpentine, peppermint, menthol, camphor, methyl salicylate, anise,

eucalyptol each more than 120 minutes, apiol and lemon more than 24 hours, and 1 per cent phenol 60 minutes.

Collier and Nitta (1930) studied the effect of essential oils on a number of species of bacteria, and reported that individual oils vary greatly in their action against different bacterial species. They studied the action of over 100 essential oils on such organisms

TABLE 43.—CRUDE ESSENTIAL OILS

<i>Crude Oil</i>	<i>Principal Active Constituent</i>	<i>Phenol Coefficient</i>
Backhousia citriodora	Citral	16
Doryphora sassafras	Safrol, camphor eugenol	13
Leptospermum citratum	Citral and citronellal	15
Santalum album	Sesquiterpene alcohols	1.5

TABLE 44.—PURE CONSTITUENTS OF ESSENTIAL OILS

<i>Constituent</i>	<i>Nature</i>	<i>Phenol Coefficient</i>
Cymene	Hydrocarbon	8
Thymol	Phenol	25
Menthone	Ketone	10
Borneol	Alcohol	10
Citronellol	"	14
Safrol	Phenol ether	11
Iso-safrol	" "	12
Eugenol	Phenol	15
Heliotropine	Aldehyde	3
Ionone	Ketone	1.75
Methyl salicylate	Ester	5.5

as streptococci, staphylococci, gonococci, *E. coli*, and a vibrio. In general they found oil of lemongrass, Ceylon cinnamon, and cassia to be the most effective. Gonococci were the most easily killed and staphylococci the most resistant.

Only two oils were effective against *E. coli* in dilutions greater than 1 to 1000, coriander up to 1 to 1600 and Ceylon cinnamon up to 1 to 4000. The oils of lemongrass, cinnamon-leaf, cinnamon, cassia, pimento, bay and thyme were effective against staphylococci in dilutions up to 1 to 1600, and Spanish hop oil up to 1 to 16,000. Against gonococci, meliassa oil was effective at dilutions from 1 to 1600 to 1 to 8000, bitter almond 1 to 4000 to 1 to 8000, Ceylon cinnamon 1 to 8000 to 1 to 16,000, lemongrass and marjoram oils up to 1 to 8000, cassia up to 1 to 16,000, and mustard oil 1 to 4000.

o 1 to 32,000. Germicidal dilutions against streptococci were found to be 1 to 1600 to 1 to 4000 for lemongrass, cedarleaf, clove, and hyeme oils, 1 to 4000 for wintergreen oil, 1 to 8000 for parsley and

TABLE 45.—PURE CONSTITUENTS OF ESSENTIAL OILS

<i>Constituent</i>	<i>Nature</i>	<i>Phenol Coefficient</i>
Linalool	Alcohol	13
Linalyl acetate	Ester	5.25
Coumarin	Lactone	4
Vanillin	"	3.5
Methyl eugenol ether	Phenol ether	13.5
Bornyl acetate	Ester	6
Amyl salicylate	"	4
Benzyl acetate	"	2
Benzyl alcohol	Alcohol	5.25
Benzaldehyde	Aldehyde	9
Anthranilic acid	Acid	2
Methyl anthranilate	Ester	6.5
Anethole	Phenol ether	11.0
Anisaldehyde	Aldehyde	7
Cinnamic aldehyde	Aldehyde	17
Menthol	Alcohol	20

TABLE 46.—ESSENTIAL OIL CONSTITUENTS AND SYNTHETIC SUBSTANCES

<i>Constituent</i>	<i>Nature</i>	<i>Phenol Coefficient</i>
Zierone	Ketone	2
Isomenthol	Alcohol	20
Hydro-cinnamic aldehyde	Aldehyde	7
Hydroxy-citronellol	"	5.3
C8-aldehyde	"	16
C9 " "	"	22
C10 " "	"	7
C11 " "	"	6
C12 " "	"	1
C13 " "	"	3
8C-alcohol	Alcohol	25
C9 " "	"	13
C10 " "	"	5

op oils, 1 to 32,000 for cinnamon oil, and 1 to 40,000 for cassia and mustard oils.

Marsh and Maus (1930) reported that the essential oils they investigated were more or less strongly germicidal against *B. anthracis*, *S. typhosa*, *E. coli*, and *Micrococcus pyogenes* var. *aureus*.



They noted also that a specific oil was often active to a varying degree on the different organisms. Oil of thyme, bay, cubeb, pimento, and cloves were the most active of those tested.

Rideal, Rideal, and Scriver (1928) and Rideal, Sciver and Richardson (1930) studied the correlation between germicidal properties of essential oils and their capillary activities, using *S. typhosa* as the test organism. Phenol coefficients using the Lancet method compared with results reported by Martindale who used the Rideal-Walker procedure are given in Table 47.

TABLE 47

<i>Oil Tested</i>	<i>Lancet Phenol Coefficient</i>	<i>R. W. Phenol Coefficient</i>
Cinnamon leaf	9.66	7.5
Clove	8.88	8.0
Cassia	5.34	1.4
Eucalyptus	4.35	1.6
Lavender	4.34	1.6
Lemon	3.94	0.4

The relative order of efficiency was the same by both methods of test, although the coefficients differ considerably. Additional phenol-coefficient data on other essential oil constituents were also presented.

TABLE 48

<i>Oil Tested</i>	<i>Chief Constituents</i>	<i>Phenol Coefficient</i>
Kachigrass	Geraniol, terpenes	3.6
Bothagrass	Sesquiterpenes, terpenes	1.5
Lemongrass	Citral, terpenes	17.0
Camphor	Camphor, safrol	6.2
Cardamom	Terpenes, borneol, cineol	10.0
Fennel	Anethol	13.0
Clove	Eugenol	5.5

Courmont, Morel, and Bay (1913, 1928, *a*, *b*, 1937) found eugenol to have the strongest action, with thymol next, against human tubercle bacilli. Oil of peppermint and menthol were intermediate in action, with guaiacol the weakest of the oils which were investigated. It was also shown that there was a weakening of the inhibitory action of essential oils towards micro-organisms in the colloidal state, being lower in colloidal than in molecular solutions.

This difference was attributed to reduced contact between the test organisms and oil when in the colloidal state.

De and Subrahmanyam (1930) studied the disinfection action of certain Indian essential oils and reported the phenol coefficients listed in Table 48.

Miller (1931) studied the bactericidal efficiency of several essential oils and mixtures of oils. The oils were tested in water, in alcohol, and in soap solutions with best results in soap solutions. Two per cent thymol killed staphylococci in 1 to 25 dilution in 2

TABLE 49

<i>Solution Tested</i>	<i>Phenol Coefficient of Solution</i>	<i>Phenol Coefficient Oil, or Active Ingredient</i>
Thymol 2%	0.55	27.6
Eugenol 4%	0.38	9.7
Beta Naphthol 2%	0.22	11.4
Menthol 4%	0.20	5.1
Cassia oil 2%	0.11	5.7

minutes, 2 per cent b-naphthol in 1 to 5 in 4 minutes, and 4 per cent eugenol in 1 to 5 in 4 minutes. The following oils killed the staphylococci in 2 minutes, but not in 1 to 5 dilutions: Eucalyptol 4 per cent, methyl salicylate 2 per cent, safrol 1 per cent, anaethol 1 per cent, menthol 4 per cent, Ceylon cinnamon 2 per cent, cinnamic aldehyde 1 per cent, cassia 2 per cent, sassafras 1 per cent, and lavender 1 per cent. Aging of the oils produced a decrease in activity in some instances, but not to any appreciable degree for cassia, cinnamon, anaethol, and methyl salicylate. Representative results are given in Table 49.

Gattefosse (1932) reported that pure oils of lavender, clove, thyme, peppermint, cinnamon and others have good antiseptic values, but, when adulterated, their activity was reduced.

Risler (1936 *a*, *b*) studied the continued effect of essential oils soaked into pieces of wood in preventing the growth of microorganisms, especially *Micrococcus pyogenes* var. *aureus*. The number of days that elapsed before growth of the staphylococci occurred in the various pieces of wood impregnated with the respective oils are shown in Table 50.

It was also noted that mixtures of oils with resins and other non-volatile substances increased the effective time considerably, even to months and years in some cases.

Blum and Fabian (1943) studied 32 spices and some compounds derived from them for use as preservatives against organ-

isms obtained from yeast vats. They found oil of mustard best for the purpose, followed by cassia, cloves, and cinnamic aldehyde.

Bose *et al.* (1949 *a, b*; 1950) using the Rideal-Walker phenol-coefficient method determined the bactericidal powers of certain essential oils and some of their constituents and showed the relative effects of the chemical nature or structure of some of these compounds on their micro-biological activity. They conclude that

TABLE 50

<i>Oil</i>	<i>Days Required for Growth</i>
Thyme	28
Lemon	20
Camphor	20
Lavender	15
Mustard	11
Cedar	8
Turpentine	4

alcohols and aldehydes in general are good germicides, while the acids and esters derived from them are not. One per cent aqueous solutions of the oils in 1 per cent potassium carbonate were used in these tests. A partial list of their results is given in Table 51.

The effect of organic matter on the germicidal activity of certain essential oils and the effect of particle size of the medicant or oil on the phenol coefficient were also reported.

Schroeder and Messing (1949) using the zone of inhibition filter-paper technique, reported on the antibacterial properties of a selected group of essential oils. The zone size was used as a measure of relative efficiencies, determining the dilutions of the oils, dissolved in a solvent such as acetone or alcohol, required to produce identical zone sizes, then listing the relative efficiencies of the oils tested on that basis. Although the suitability of the method of test is open to question and while the results obtained do not accurately reflect the effectiveness of the oils tested, the inhibitory activity of these oils is nevertheless of some interest. Oils of cassia and cinnamon, at 1 to 40 and 1 to 100 dilutions, were observed to be bacteriostatic against 11 of the 15 organisms tested. Oil of pine had greater activity against gram-negative organisms than against the gram-positives. Cinnamic aldehyde was effective in 1 to 40 dilution against all organisms tested except *B. subtilis* and *Ps. aeruginosa* while cinnamic acid showed very low activity, requiring 1 to 2



and 1 to 4 dilutions. Benzaldehyde was not inhibitory at 1 to 10, 1 to 20, and 1 to 100 dilutions. Hydrocinnamic aldehyde was more active than cinnamic aldehyde, being effective against all organisms in 1 to 40 dilution. The type of solvent in which the oils were dissolved made little difference as far as zone size was concerned.

Okazaki *et al.* (1950, 1951, 1952) conducted extensive studies on the antibacterial activity of higher plants. Many types of test organisms were used, both bacteria and fungi. The activity of clove

TABLE 51

<i>Oil of Constituent Tested</i>	<i>Phenol Coefficient</i>
Citral . . . . .	20.0
Geraniol . . . . .	15.5
Citronellol . . . . .	12.5
Citronellal . . . . .	8.0
Citronellic acid . . . . .	less than 1
Cinnamic aldehyde . . . . .	16.5
Cinnamic alcohol . . . . .	11.0
Cinnamic acid . . . . .	2.5

oil and eugenol against 15 organisms was reported and also the activity of chenopodium oil and ascaridol against athlete's foot fungi. Bacteria such as *Micrococcus pyogenes* var. *aureus*, *M. tuberculosis*, and *Brucella* were used, as well as such fungi as *Trichophyton*, *Achorium* and *Epidermophyton*. Inhibitory activity for clove oil and eugenol against fungi was found to be in the range of 1 to 8000 to 1 to 16,000, while for chenopodium and ascaradol oil approximately 1 to 6400. The antibacterial activity of over 100 crude drugs listed in the Japan Pharmacopeia against *Micrococcus pyogenes* var. *aureus* was determined. Approximately 50 per cent of the plants tested were found to be active.

Lilley and Brewer (1953) described the selective antibacterial action of phenylethyl alcohol, and reported effective inhibitory activity against *E. coli* and less effective against *Micrococcus pyogenes* var. *aureus*. The compound was also found to be fungistatic against 15 of the 16 molds and yeasts used in the tests.

Much other research on essential oils has been reported which cannot be discussed because of space limitations. Some of these publications are of sufficient interest to be listed in the bibliography, even though they have not been included in the review. This serves further to complete the bibliography on this important subject.

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## 14

### ALCOHOLS

As a chemical group the alcohols possess many desirable features for a disinfectant. They have a bactericidal rather than a bacteriostatic action against vegetative forms, they are relatively inexpensive and usually easily obtainable. They have a cleansing action and readily evaporate. They are colorless but if this property is undesirable a coloring substance may be added. Like chemical disinfectants in general their destructive action against spore forms is much less than that against vegetative forms. They have been studied more than any other group of disinfectants but they are far from being clearly understood. By far the greatest amount of work has been done with ethyl alcohol.

Wirgin (1904) pointed out that insofar as methyl, ethyl, propyl, butyl, and amyl alcohol were concerned, the bactericidal action of the aliphatic alcohols increased with the increase in the molecular weights of the alcohols, with the exception of the tertiary alcohols. Testing alcohols in a liquid environment against *Salmonella typhosa* Tilley and Schaffer (1926) observed an increase in the phenol coefficients from 0.026 for methyl to 21.0 for octyl alcohol. For *Micrococcus pyogenes* var. *aureus* the phenol coefficients varied from 0.030 for methyl to 0.63 for amyl alcohol. Tanner and Wilson (1943) tested alcohols containing from 1 to 11 carbon atoms by the agar cup plate method. Methyl alcohol had no noticeable effect on the test organisms. The size of the zones in which no growth of the test organisms took place increased in size from that for ethyl alcohol to that for amyl alcohol, then decreased in size for the primary-normal alcohol series. The size of the zone of inhibition for primary-normal undecyl alcohol, the last alcohol in the series to be tested, was comparable to that for ethyl alcohol. In the case of *Pseudomonas aeruginosa*, growth was stimulated rather than inhibited by the sample of undecyl alcohol. The bactericidal action varied from greatest to lesser in the following order: primary-



normal > primary-iso- > secondary-normal > and tertiary alcohols, as was observed by Tilley and Schaffer (1926). Nine months contact with 100 per cent primary-normal methyl, ethyl, propyl, butyl, amyl, hexyl, heptyl, octyl, nonyl, and undecyl alcohols did not kill *Bacillus subtilis* and *Bacillus megatherium*.

The bactericidal action of alcohols is important because frequently proprietary preparations are marketed as alcoholic solutions. Rodriguez (1928) and Reddish and Drake (1928) demonstrated that the alcohol-acetone solution of mercurochrome was more effective than the aqueous solution. Molinas and Brewer (1949) studied 27 compounds for their ability to reduce the number of bacteria on rabbit, guinea pig, and human skins. Solutions of 50 per cent ethyl alcohol and 10 per cent acetone or 50 per cent iso-propyl alcohol and 10 per cent acetone were used as controls. These investigators came to the conclusion that the action of proprietary preparations designated for disinfecting the skin is largely dependent upon the alcoholic solutions in which they are incorporated. The results of *in vitro* tests with fewer compounds reported by Simmons in 1928 indicated the same thing. In quantitative tests Novak and Hall (1939) observed the 50 per cent alcohol-10 per cent acetone mixture to be highly germicidal. It killed 96 per cent of the organisms on the skin, whereas no disinfectant tested was able to kill 100 per cent of the organisms and tincture of green soap was the poorest, killing only 53 per cent of the organisms. The tests were performed on the skin over the abdomen where, on the average, 2 staphylococci and diphtheroids were found per sq cm of skin.

MODE OF ACTION OF ALCOHOLS.—(a) *Denaturing Action*.—The most plausible explanation for the antibacterial action of the alcohols is their denaturing of proteins. In the absence of water proteins are not denatured as readily as when water is present. This affords an explanation of why absolute ethyl alcohol, a dehydrating agent, is less bactericidal than mixtures of alcohol and water.

(b) *Interference with Metabolism*.—Dagley, Dawes, and Morrison (1950) observed that a 0.41 molar concentration of ethyl alcohol increased the lag phase of *Aerobacter aerogenes*. This effect was decreased by the presence of the amino acids dl-methionine, leucine, l-glutamic acid, l-histidine and dl-tryptophane. The lag was increased by l-proline, glycine, dl-alanine, dl-serine, and dl-aspartic acid. The authors concluded that the bacteriostatic action was due to the inhibition of the production of metabolites essential for rapid cell division.

(c) *Lytic Action*.—Pulvertaft and Lumb (1948) reported that lysis of microorganisms occurred with many antiseptics when

used at a concentration approximately double that of the minimum concentration producing bacteriostasis. Lysis was observed with formalin, mercuric chloride, merthiolate, phenol, and sodium hypochlorite, as well as with the antibiotic penicillin. Lysis was most marked with staphylococci, pneumococci, *B. subtilis* and *Escherichia coli*, less marked with Flexner and Sonne strains of dysentery bacilli, and only very slight with streptococci. The organisms were most susceptible to lysis when the antiseptic was added to the culture during its logarithmic phase and lysis usually set in about the third hour thereafter. The lytic action was thought possibly to be due to using the antiseptic in a concentration which inhibited growth of the microorganisms without inhibiting the autolytic enzymes.

Lecce (1953) observed that a suspension of the Campo strain of pleuropneumonia-like organisms isolated from man, estimated to contain 140 mcg nitrogen per ml, was lysed within a few minutes by tertiary-butyl and iso-amyl alcohols in a concentration of 0.1 per cent. The organisms were not lysed under similar conditions by methyl, ethyl, propyl, iso-propyl and normal butyl alcohols. *E. coli* in a suspension estimated to contain 500 mcg dry weight per ml was lysed in the presence of 33 per cent propyl and tertiary-butyl alcohols.

### METHYL ALCOHOL

Methyl alcohol has the weakest bactericidal action of the alcohols so is seldom considered for an antibacterial agent. Its slight action was noted by Wirgin (1904) and Tilley and Schaffer (1926) although Tanner and Wilson (1943) observed no noticeable effect. It apparently does not noticeably affect the bactericidal action of ethyl alcohol when used as a denaturant (Olivo, 1948).

### ETHYL ALCOHOL

Alcohol is widely used for the destruction of the vegetative forms of microorganisms before such procedures as venipunctures, hypodermic injections, finger pricks to obtain blood for blood counts and hemoglobin determinations and other procedures which break the intact skin. During the latter part of the last century it was common practice to test disinfectants against microorganisms dried on threads or beads. This brought out the fact that some water must be present for alcohol to exert its most effective bactericidal action. It also gave the erroneous impression that alcohol is a poor disinfectant because it failed to kill readily the dried organisms and, like chemical disinfectants in general, it possessed poor killing

action against bacterial spores. The erroneous statement was perpetuated in many textbooks that 95 per cent alcohol was practically worthless as a disinfectant, possibly because 70 per cent alcohol possessed a stronger killing action. In 1939, Price attempted to correct the erroneous impression that alcohol was a poor disinfectant.

Harrington and Walker (1903) tested organisms on moist and dry threads against various concentrations of alcohol. *E. coli* on moist threads was killed in less than 5 minutes exposure to alcohol of 60 to 99 per cent concentrations whereas only the concentrations of 50 and 60 per cent killed within 5 minutes when the organisms were dried on threads. Concentrations of 94 and 99 per cent did not kill *E. coli*, *P. aeruginosa*, and *Micrococcus pyogenes* var. *albus* and *aureus* in an exposure of 24 hours when the organisms were dried on threads. *P. aeruginosa* on moist threads was killed in 5 minutes by concentrations of 40 to 99 per cent but on dried threads only the concentrations of 40 to 70 per cent killed in the same period of time. *M. pyogenes* var. *albus* on moist threads was killed in less than 1 minute by 50 per cent alcohol. Concentrations of 40 to 99 per cent killed in 5 minutes but when the organisms were dried on threads only the concentrations of 40 to 70 per cent killed in the same period of time. Practically the same results were obtained with *M. pyogenes* var. *aureus*. *S. typhosa* on moist threads was killed in 5 minutes by concentrations of 30 to 99 per cent but on dried threads only by concentrations of 30 to 80 per cent. *Corynebacterium diphtheriae* on moist threads was killed in 5 minutes by concentrations of 40 to 99 per cent and on dried threads by concentrations of 40 to 90 per cent. These authors concluded half a century ago that against the commoner, non-sporing, pathogenic bacteria in a moist condition, any strength of alcohol above 40 per cent by volume is effective within 5 minutes and certain preparations are effective within an exposure time of one minute. Against organisms in the dry state concentrations of 60 to 70 per cent are the most effective and they are equally effective against the organisms in the moist state. Typical results are reproduced in Table 52.

By adding a loopful of suspension of microorganisms to the solution of disinfectant Post and Nicoll (1910) found that 70 and 60 per cent concentration of alcohol killed *Streptococcus pyogenes*, *Diplococcus pneumoniae*, *Neisseria gonorrhoeae* and *S. typhosa* in less than 1 minute. Even 30 per cent alcohol killed *N. gonorrhoeae* in less than 1 minute.

Morton (1950) tested various concentrations of alcohol against a variety of microorganisms in exposure periods beginning with 10 seconds. In order not to alter the concentrations of the alcohol,







the test organisms in 0.5 ml amounts of broth culture were placed in sterile tubes and centrifuged. The supernatant was removed and the alcoholic solution was thoroughly mixed with the moist cells. One drop of the culture-cell mixture was subcultured at stated intervals. *P. aeruginosa* was killed in 10 seconds by all concentrations of alcohol from 100 to 30 per cent by volume. The lowest concentration of alcohol tested, 20 per cent, killed in 30 minutes. *Serratia marcescens*, *E. coli*, and *S. typhosa* were killed in 10 seconds by all concentrations of alcohol from 100 to 40 per cent. Nathanson (1951) stated that 2 per cent alcohol added to their products (sulfonated vegetable oils) prevented the growth of *S. marcescens* and *P. aeruginosa* in most cases. The gram-positive vegetative organisms, *M. pyogenes* var. *aureus* and *S. pyogenes* were a little more resistant, being killed in 10 seconds by concentrations of 60 to 95 per cent. Exposures of 50 and 90 seconds, respectively, were required to kill the two organisms with absolute alcohol. Table 6 from Morton (1950) is reproduced as Table 53. The temperature of medication was room temperature. At higher temperatures the killing action would be expected to be more rapid (Tilley, 1942). Price (1939) employed an original technique wherein the alcohol-culture mixture was diluted with water to stop the germicidal action, aliquot portions seeded into plates and colonies from surviving organisms counted. He observed *E. coli* to be killed in less than 60 seconds by 60 per cent alcohol. (by weight), in less than 40 seconds by 70 per cent and in less than 30 seconds by 80 per cent alcohol. *M. pyogenes* var. *albus* was killed in less than 60 seconds by either 50 or 70 per cent alcohol. *M. pyogenes* var. *aureus* was killed in less than 15 seconds by 70 per cent alcohol. However, by employing essentially the same technique, Price (1950) reported that *E. coli* required an exposure of 5 minutes in order to be killed by 60 per cent alcohol, *M. pyogenes* var. *aureus* required a similar exposure to be killed by either 70 or 80 per cent and *M. pyogenes* var. *albus* was not killed in 10 minutes by any concentration of alcohol.

Seelig and Gould (1911) appear to have been annoyed somewhat at the earlier methods of testing germicides for they stated that in the animal body bacteria do not occur in a state of acrobatic suspension on strings, nor are they found even in the better class of patients adherent to garnets, or rarely are they found under such conditions as to enable them to be effectually bathed in a solution. Microorganisms are usually found in the deeper layers of the skin and tissues or covered with blood or exudate, so for practical purposes a disinfectant must possess power of penetration. To test the power of penetration the authors made a pouch of cel-



oidin or living animal tissue at the end of a glass tube, placed the culture under test inside of the pouch and immersed it into the disinfectant solution. After varying periods of time sub-cultures were made of the culture within the pouch. *S. typhosa* within celloidin pouches was killed within 6 minutes by 95 and 99.8 per cent alcohols. Concentrations of 50, 70 and 80 per cent did not kill in 1 hour. In a pouch of living rabbit omentum immersed in 95 per cent alcohol the organisms were killed within 3 minutes. In a pouch of living rabbit skin immersed in 95 per cent alcohol the organisms were alive after 5 minutes but dead after 10 minutes. When placed in a pouch of dried rat intestine and immersed in 99 and 95 per cent alcohols the organisms were still alive after 18 hours. An important incidental observation was that when the organisms lost their motility they were no longer viable. By making microscopic observations of the organisms inside the pouches they could determine if the organisms had been killed and the results of subculturing verified this in every case. In celloidin pouches *E. coli* was killed in 20 but not in 10 minutes by 95 per cent alcohol, in 60 but not in 30 minutes by 80 per cent, in 18 hours but not in 2 hours by 70 per cent, and 50 per cent alcohol did not kill in an exposure of 18 hours. Comparable results were obtained with a staphylococcus culture. The authors stated that the power of penetration of 95 per cent alcohol was put to satisfactory use in the treatment of a case of furunculosis. If a pack saturated with 95 per cent alcohol was applied early enough over a furuncle, the furuncle was absorbed. When certain germicides were dissolved in alcohol their germicidal action was increased. An alcoholic solution of iodine killed micro-organisms within the pouches quicker than 95 per cent alcohol or aqueous solution of iodine alone.

In testing the effect of ethyl alcohol against *Mycobacterium tuberculosis* Smith (1947) observed that 95 per cent alcohol killed the tubercle bacilli in sputum within 15 seconds, absolute and 70 per cent alcohol required 30 seconds and 50 per cent alcohol required 60 seconds. Tubercle bacilli in water suspensions were killed in 15 seconds by 95 per cent alcohol, in 30 seconds by absolute alcohol and in 60 seconds by 70 per cent alcohol. When tuberculous sputum was allowed to dry on cover slips and then subjected to the action of the alcohols 50 per cent alcohol killed the tubercle bacilli in 1 or 2 minutes, depending on the thickness of the smears. In thin smears 70 per cent alcohol killed the organisms in 1 minute but in thick smears the organisms were not killed in 5 minutes. In thick smears 95 per cent killed the organisms in 30 minutes and absolute alcohol did not kill in an exposure of 60 minutes. Practically the same results were obtained with tubercle bacilli dried from water

suspensions. He concluded that 95 per cent ethyl alcohol was best for wet surfaces, 50 per cent for dry surfaces, and 70 per cent for either wet or dry surfaces. The number of tubercle bacilli has an effect upon the killing action of 95 per cent alcohol. When 0.0001 or 0.1 mgm of bacilli were used some of the subcultures were sterile after the organisms had been exposed for 2 minutes and all subcultures from the longer exposures were sterile (Cohn, 1934).

Like the majority of chemical disinfectants alcohol appears to be more toxic for certain tissue cells than for bacterial cells. Welch and Brewer (1942) found absolute alcohol to be 7.5 times more toxic for the phagocytic cells of human and guinea pig blood than for *M. pyogenes* var. *aureus*.

*Effect of Ethyl Alcohol on Bacterial Spores.*—It is well known that ethyl alcohol has little effect against bacterial spores. Coulthard and Sykes (1936) demonstrated that the bactericidal action of ethyl alcohol was greatly increased by the addition of 10 per cent amyl-meta-cresol, or 1 per cent hydrochloric, nitric, phosphoric, or sulfuric acid or sodium or potassium hydroxide. They stated that the bactericidal action of methyl and iso-propyl alcohols could be similarly enhanced. That samples of alcohols of various concentrations occasionally contain bacterial spores cannot be denied. Alcohol may become contaminated with spores from having contaminated materials immersed in it. Kuhn and Dombrowsky (1932) reported half of the samples of alcohols tested to be contaminated. Gershenfeld (1938) tested 100 samples of 95 per cent alcohol and 25 samples of absolute alcohol obtained on the open market from many different sources in the United States and found all to be free of bacteria and bacterial spores.

That fatal infections with *Clostridium* may result when depending upon alcohol for sterilization of surgical instruments contaminated with bacterial spores was demonstrated by Nye and Mallory in 1923. Saegesser (1941) demonstrated gas gangrene bacilli in 70 per cent alcohol in which syringes were stored, and emphasized that alcohol was not an adequate method for sterilization.

*Effect of Ethyl Alcohol on Viruses.*—Vaccinia virus was reported by Gordon (1925) to be inactivated by 50 per cent ethyl alcohol in one hour but not by 20 per cent alcohol in 24 hours. Methyl alcohol appeared to be slightly more effective in that a 20 per cent concentration had a noticeable effect in one hour and inactivated the virus in 24 hours. It is not surprising that Dunham and MacNeal (1943) found that 25 per cent ethyl alcohol did not inactivate vaccinia virus in an exposure of 3 minutes. The virus of Newcastle disease was observed by Cunningham (1948) to be

inactivated by 70 and 95 per cent alcohols in an exposure period of 3 minutes. Alcohols of 40 and 25 per cent concentrations did not inactivate the virus under similar conditions. The results with 25 per cent ethyl alcohol were comparable to those with iso-propyl alcohol and the same virus as reported by Tilley and Anderson (1947).

When mixed with an equal volume of saline filtrate of the virus of hoof-and-mouth disease and held at room temperature, alcohol did not inactivate the virus in 3 days but did in 6 days (Stockman and Minett, 1926).

The danger of transmitting hepatitis virus by transferring material from one to another has become apparent in recent years. The use of a syringe and needle on more than one individual without proper sterilization between individuals is now recognized as a hazardous procedure. The use of a blood lancet or knife blade for obtaining small amounts of blood must be recognized, likewise, as a procedure not without risk. Dipping a blood lancet into alcohol and allowing it to dry or wiping a knife blade with a pad moistened with alcohol should not be considered as a safe procedure until there is published information on the effect of alcohol on these viruses. At present there is circumstantial evidence of epidemiological nature that such procedures may be unsafe.

*Ability of Ethyl Alcohol to Prevent Infection.*—The crucial test of any disinfectant is the ability of the substance to prevent infection in a susceptible animal with the test organism. Neufeld and Schiemann (1938) demonstrated by intraperitoneal injection in mice that a culture of *D. pneumoniae*, type 1, on the finger tips was killed by treating with 80 per cent alcohol. Not all of the pneumococci were destroyed when 96 per cent alcohol was used.

Nungester and Kempf (1942) devised a test wherein the tail of a mouse was scrubbed with 4 or 5 strokes of a swab which had been moistened with a broth culture of the test organism. The tail was then dipped into a tube containing the disinfectant for 2 minutes. At the end of this time a half-inch portion of the tail was cut off and implanted in the peritoneum of the same animal. In the case of *D. pneumoniae*, type 1, 100 per cent of the mice died following treatment of their contaminated skin with either physiological salt solution, which served as a control, or aqueous merthiolate, 1:1000. There was a mortality of 69 per cent among the mice when a mixture of 50 per cent alcohol and 10 per cent acetone was used and 63 per cent mortality when 70 per cent alcohol (by volume) was employed. Employing 2 per cent aqueous iodine solution resulted in a mortality of only 5 per cent but when a 2 per cent iodine solution in 95 per cent alcohol was employed the mortality was



zero. The alcohol in the alcoholic solution of iodine enhanced the bactericidal efficiency of this solution. In the case of some mice a portion of tail was also cultured. In general, more animals died of the infection than growth was obtained in the subcultures. When using a hemolytic streptococcus none of the animals were protected by the use of the alcohol-acetone mixture.

Employing the technique of Nungester and Kempf and *D. pneumoniae*, Prombo and Tilden (1950) obtained a mortality of slightly more than 10 per cent when using 70 per cent alcohol by weight and slightly less than 20 per cent when using 70 per cent alcohol by volume. With *S. pyogenes* and 70 per cent alcohol by weight there was a 20 per cent mortality in the mice.

One would not expect that 1:5,000 and 1:10,000 dilutions of 70 per cent ethyl alcohol, as used by Murphy *et al.* (1951), would have any protective action *in vivo*.

*Ethyl Alcohol in Skin Degermation.*—It is nearly impossible to sterilize the skin. The best one can hope to accomplish is to reduce the number of viable organisms on or in the skin and to destroy the pathogenic organisms which may be on the skin as transients. The term "degermation" is more accurate than "disinfection" (Price, 1939). He pointed out that, in the surgical scrub, immersion of the hands and arms in a 65.5 per cent solution of alcohol by weight for 1 minute was as effective as scrubbing for 4.2 minutes as far as reducing the number of bacteria on the skin is concerned. This effectiveness of alcohol appears to have been amply confirmed (Pohle and Stuart, 1940; Pillsbury *et al.*, 1942; Hatfield and Lockwood, 1943).

Employing a testing procedure somewhat similar to that of Price, Hatfield and Lockwood (1943) came to the conclusion that ethyl alcohol in strengths of 95 or 70 per cent by weight was preferable to any of a group of commercially prepared agents specifically designed for skin sterilization at that time. The ideal concentration was found to be 95 per cent but for economy the concentration could be reduced to 70 per cent. Pillsbury *et al.* (1942) also observed that 80 per cent or more by volume produced a satisfactory decrease in the number of viable bacteria remaining on or in the skin. They expressed doubt about the necessity of alcohol of an exact concentration for clinical use so long as the concentration exceeds 70 per cent by volume. Reddish and Drake (1928) demonstrated that 85 per cent alcohol brought about greater reduction of bacteria on the skin than did aqueous-alcohol-acetone mixture or 50 per cent alcohol.

It must be remembered that since the above publications hexachlorophene (G-11) has been introduced and has become widely

used. Its apparent advantages have been pointed out by Nungester *et al.* (1949), Dull *et al.* (1950), and Artz *et al.* (1951). Tincture of acrizane has been proposed by Murphy *et al.* (1951).

*Ethyl Alcohol for the Chemical Sterilization of Instruments.*—Some instruments, especially those with sharp cutting edges, do not lend themselves well to sterilization by heat, so "cold" sterilization by means of a chemical solution is often attempted. The ability of a chemical solution to sterilize instruments certainly can be no better than the germicidal action of the solution *in vitro*. Indeed, its ability to sterilize instruments may be lessened because of the rough surfaces or joint on some instruments which offer potential harboring sites for microorganisms. Contaminated instruments frequently are soiled with blood, pus or other body fluids which make bacteria more difficult to destroy. The inability of alcohol, like other chemical disinfectants, to destroy bacterial spores makes this method of sterilization hazardous. In the absence of bacterial spores, alcohol is as effective for sterilizing instruments as its germicidal action is indicated by *in vitro* bactericidal and infection-prevention tests, namely, it is better than many substances but is not 100 per cent reliable. In its ability to sterilize Bard-Parker knife blades contaminated with pus or blood, wet or dry, Spaulding (1939) observed that 70 per cent alcohol killed *Monilia albicans*, *E. coli*, *S. pyogenes*, and *Ps. aeruginosa* in either one-half or one minute exposure, rarely was an exposure of 2 minutes required. *S. pyogenes* var. *aureus* was slightly more resistant when the pus and blood were dried. Ten minutes was required to kill the organisms in dried pus and 5 minutes in dried blood. *Cl. tetani*, *Cl. perfringens*, and *B. anthracis* were not killed after an exposure of 18 hours except *B. anthracis* in wet pus which was killed in that period of time.

*Sterilization of Clinical Thermometers.*—Force and Kerr (1920) gave serious thought to the necessity for sterilization of clinical thermometers. Because of their construction clinical thermometers are best sterilized by chemical disinfectants. Since alcohol is non-poisonous, colorless, nearly tasteless, readily evaporates, and leaves no residue, it was selected for the sterilizing agent. These investigators reported that an exposure of 4 minutes to 50 per cent alcohol would secure adequate disinfection of oral thermometers, if the thermometers were wiped with a cotton sponge wet with water to free them of mucus before placing them in the alcohol. Results of two rather extensive investigations were published recently. The results of Sommermeyer and Frobisher (1952) are reproduced in Table 54. Glass rods or unfilled mouth thermometers were contaminated with a thin film of tuberculous sputum

which was strongly positive for acid-fast bacilli by direct smear examination. Cultures of *C. diphtheriae* were added to the samples of sputum which contained staphylococci, streptococci and other bacteria in addition to the acid-fast bacilli. The contaminated

TABLE 54. — COMPARISON OF DISINFECTANTS USED IN TEST PROCEDURES FOR STERILIZING CLINICAL THERMOMETERS. (FROM SOMMERMEYER AND FROBISHER, 1952.)

Disinfectant (exposure time 10 minutes)	Tests (rods <sup>1</sup> and thermometers)	Cultures positive for			
		Streptococci, staphylococci or diphtheria bacilli		Tubercle bacilli	
		Number	Per cent	Number	Per cent
Aqueous Quaternaries 1:1,000 (A and B) <sup>2</sup>					
without wiping	50	—	—	50	100 0
with dry wiping	50	—	—	50	100 0
with soap wiping	149	—	—	82	55 0
Tincture of Quaternaries 1:1000 (A and B)					
without wiping	50	—	—	0	0 0
with dry wiping	50	—	—	0	0 0
with soap wiping	150	—	—	0	0 0
Ethyl Alcohol 70 per cent					
without wiping	30	0	0 0	2	6 6
with dry wiping	225	4	1 8	9	4 0
with soap wiping	424	17	4 0	3	0 7
Isopropyl Alcohol 70 per cent					
without wiping	60	6	10 0	7	11 6
with dry wiping	15	9	60 0	11	73 3
with soap wiping	224	3	1 3	2	0 9
Ethyl Alcohol, 70 per cent, and Iodine <sup>3</sup>					
without wiping	390	17	4 4	28	7 2
with dry wiping	565	8	1 4	1	0 2
with soap wiping	998	9	0 9	4	0 4
Isopropyl Alcohol, 70 per cent and Iodine <sup>4</sup>					
without wiping	470	12	2 6	16	3 4
with dry wiping	370	6	1 6	5	1 4
with soap wiping	1048	10	1 0	5	0 5
Aqueous KI 1 per cent with Iodine <sup>5</sup>					
without wiping	300	36	8 7	40	13 3
with dry wiping	250	2	0 8	1	0 4
with soap wiping	500	8	1 6	3	0 6
Formalin, 4 per cent and 10 per cent					
without wiping	50	44	88 0	35	70 0
with dry wiping	200	67	33 5	89	44 5
with soap wiping	400	45	11 3	120	30 0

<sup>1</sup> Glass rods were used in place of thermometers in some of the tests.

<sup>2</sup> Cetyl-pyridinium chloride and alkyl-dimethyl-benzyl-ammonium chloride used.

<sup>3</sup> Three concentrations of iodine, 0 05, 0 25, and 1 per cent were tested. The one per cent concentration appeared more effective but only a few tests were done with it. The results with the three concentrations of iodine are combined in the table.

<sup>4</sup> Concentrations of 0 05 and 0 25 per cent iodine in 70 per cent isopropyl alcohol yielded only an occasional positive culture. When the concentration of iodine was increased to 0 5 and 1 per cent, all subcultures were negative for the test organisms. These higher concentrations of iodine in isopropyl alcohol were found irritating to the eyes of the workers and for that reason iodine in ethyl alcohol solutions may be preferable.

<sup>5</sup> Concentrations of iodine of 0 05, 0 25 and 1 per cent were used.



thermometers were allowed to dry for 30 minutes before applying the sterilizing procedures. The contamination probably was excessive of that encountered in ordinary practice, so the results possibly err on the side of safety. These authors found that wiping the thermometers with cotton and a mixture of equal parts of 95 per cent ethyl alcohol and tincture of green soap before placing in the disinfectant decreased the number of viable organisms in practically every case. After the cleaning procedure, immersion of the thermometers for 10 minutes in 70 per cent ethyl or iso-propyl alcohol containing 0.5 or 1 per cent iodine reduced the number of viable organisms to a very low number. Aqueous iodine solutions or 70 per cent solutions of ethyl or iso-propyl alcohol were nearly as effective as the alcoholic iodine solutions. The importance of wiping the thermometers was emphasized by Ryan and Miller (1932).

Gershenfeld, Greene, and Witlin (1951) tested various disinfectants for their ability to sterilize 2 inch segments of clinical thermometers contaminated with bacterial cultures. Their results are summarized in Table 55. Ethyl alcohol, 95, 70, and 50 per cent concentrations, iso-propyl alcohol, 70 and 50 per cent and 2 per cent iodine, aqueous solution and tincture, were tested. *D. pneumoniae* was killed by all disinfectants within 20 seconds. *S. pyogenes* was killed by all disinfectants within 20 seconds except 95 per cent ethyl alcohol, which required 80 seconds. Cultures of these two organisms were used to contaminate the thermometer segments and, without drying, the segments were transferred to the disinfectants. This may be one factor which accounts for the best killing action in the experiments. An article by Ecker and Smith (1937), which appears to have been overlooked, reported that thermometers contaminated with sputum from cases of lobar pneumonia were not sterilized by exposures for as long as 30 minutes to 70 and 95 per cent solutions of ethyl alcohol. This is difficult to interpret in light of the findings of Sommermeyer and Frobisher (1952) and of Gershenfeld *et al.* (1951) with pure cultures of *D. pneumoniae*. The sputum may have exerted a protective action as Gershenfeld, *et al.* observed for plasma. It must be borne in mind that the work of Ecker and Smith (1937) was carried out prior to the general practice of neutralizing the bacteriostatic action of mercury in the subculture medium. This might account for the apparent quick sterilization of the thermometers by the mercurial compounds. In the case of the other organisms tested by Gershenfeld *et al.* (1951), the contaminated segments were thoroughly dried in an incubator at 37°C. This might account for delayed killing action. Price (1950) reported *M. pyogenes* var. *aureus* killed in an exposure of 5 minutes to either 70 or 80 per cent ethyl alcohol. Morton (1950)

reported *E. coli* killed in an exposure of 10 seconds to concentrations of ethyl alcohol ranging from 40 to 100 per cent and *M. pyogenes* var. *aureus* was killed in a similar period by concentrations of 60 to 95 per cent by weight. The latter two authors employed

TABLE 55.—COMPARISON OF DISINFECTANTS AGAINST VARIOUS MICROORGANISMS IN THE STERILIZATION OF CLINICAL THERMOMETERS. (SUMMARIZED FROM VARIOUS TABLES BY GERSHENFELD, GREENE, AND WITLIN, 1951.)

Time of immersion of thermometer in disinfectant	Disinfectant						
	Solution iodine 2%	Tincture iodine 2%	Ethyl alcohol w/v 95%	Ethyl alcohol w/v 70%	Ethyl alcohol w/v 50%	Isopropyl alcohol w/v 70%	Isopropyl alcohol w/v 50%
<i>Diplococcus pneumoniae</i>							
Control	+	+	+	+	+	+	+
20 seconds	0	0	0	0	0	0	0
<i>Streptococcus hemolyticus</i>							
Control	+	+	+	+	+	+	+
20 seconds	0	0	+	0	0	0	0
60 seconds	0	0	0	0	0	0	0
80 seconds	0	0	0	0	0	0	0
<i>Streptococcus fecalis</i>							
Control	+	+		+	+	+	+
20 seconds	+	+		+	+	+	+
40 seconds	+	0		+	+	+	+
80 seconds	+	0		+	+	+	+
100 seconds	0	0		+	+	+	+
<i>Escherichia coli</i>							
Control	+	+	+	+	+	+	+
80 seconds	+	+	+	+	+	+	+
100 seconds	0	0	+	+	+	+	+
120 seconds	0	0	+	0	0	0	0
<i>Staphylococcus aureus</i>							
Control	+	+	+	+	+	+	+
60 seconds	+	+	+	+	+	+	+
80 seconds	+	0	+	+	+	+	+
120 seconds	0	0	+	+	+	+	+
3 minutes	0	0	+	+	+	+	+
4 minutes	0	0	+	+	+	0	+
5 minutes	0	0	+	+	+	0	+
10 minutes	0	0	+	0	0	0	0
<i>Staphylococcus aureus</i> , 50% citrated human plasma-culture mixture							
Control	+	+		+	+	+	+
4 minutes	+	+		+	+	+	+
5 minutes	0	+		+	+	+	+
10 minutes	0	+		+	+	+	+
+ Growth in 48 hours				0 No growth in 48 hours			

liquid cultures. *Streptococcus fecalis* was not killed in 2 minutes exposure to the alcohols. It was killed by 40 seconds exposure to the tincture of iodine and by 100 seconds by the aqueous iodine solution. Both the tincture and aqueous solutions of iodine killed *E. coli* in 100 seconds but not in a shorter period. The alcohols, with the exception of 95 per cent ethyl, killed *E. coli* in an exposure of 2 minutes.

On the basis of his work with alcohols on *M. tuberculosis* Smith (1947) stated that if thermometers are kept immersed in 70 per cent alcohol, either ethyl or iso-propyl, they should remain non-infectious if the alcohol is changed often enough to keep its strength within the effective range.

*Denatured Alcohol.*—Ethyl alcohol (96 per cent) denatured by the addition of 10 per cent methyl alcohol was found by Olivo (1948) to have practically the same bactericidal power as ethyl alcohol. His tests were made with *M. pyogenes* var. *aureus* and *E. coli*.

## ISO-PROPYL ALCOHOL

The propyl alcohols (normal-propyl and iso-propyl) occupy an enviable position in that they are the alcohols of the highest molecular weight which are miscible with water in all proportions. Tanner and Wilson (1943) found normal-propyl alcohol to be the strongest bactericide of the water soluble alcohols. Because ethyl alcohol is subject to restrictions and heavy taxation and is important in many manufacturing processes, a suitable substitute has been sought for it as an antiseptic. Iso-propyl alcohol appears to fill this need. In a review of the literature on iso-propyl alcohol Grant (1923) pointed out that it had no noticeable harmful effect on the human skin although it is slightly more toxic than ethyl alcohol. As might be expected, its bactericidal action is slightly greater than that of ethyl alcohol. By making counts of surviving organisms after 30 seconds exposure to varying concentrations of alcohols, Coulthard and Sykes (1936) found iso-propyl alcohol slightly more bactericidal than either ethyl or methyl alcohol for *E. coli* and *M. pyogenes* var. *aureus*. The alcohols had little effect against bacterial spores.

Powell (1945) reported that *M. pyogenes* var. *aureus* was killed by a 1-minute exposure at 20°C to 50, 60, 70, 80, and 91 per cent iso-propyl alcohol solutions but not by 20, 30, and 40 per cent solutions. Other tests showed that the same organism was killed in 5 minutes by 40 per cent and greater concentrations of iso-propyl alcohol but not by 10, 20, or 30 per cent solutions. *E. coli* was killed in 5 minutes at 20°C by 30 per cent and greater concentrations of iso-propyl alcohol but not by 10 or 20 per cent solutions. Well-spored cultures of *B. subtilis* and *Clostridium novyi* were not killed in 60 minutes at 20°C by any concentration of iso-propyl alcohol ranging from 20 to 91 per cent. In tests performed some 10 years earlier Powell stated that the stocks of iso-propyl alcohol were contaminated with a saprophytic spore-forming organism, which further substantiates its ineffectiveness, against bacterial spores.



Tainter, Thronsdon, Beard, and Wheatlake (1944) reported that *M. pyogenes* var. *aureus* was killed in less than 10 seconds by a 50 per cent aqueous solution of iso-propyl alcohol. A 90 per cent solution failed to kill the organisms in an exposure of 2 hours, thus emphasizing the importance of the presence of water for effective bactericidal action as in the case of ethyl alcohol.

Against tubercle bacilli in dried sputum smears, Smith (1947) observed that the bactericidal activity of iso-propyl alcohol paralleled that of ethyl alcohol in the upper and middle ranges of concentrations but surpassed that of ethyl alcohol in the range of lower concentrations.

In preventing infection in mice with *D. pneumoniae* Prombo and Tilden (1950) found iso-propyl alcohol (99 per cent) as effective as ethyl alcohol, 70 per cent by weight, and nearly as effective as ethyl alcohol, 70 per cent by volume. It was less effective than ethyl alcohol, 70 per cent by weight, in preventing infection in mice with *S. pyogenes*.

Tilley and Anderson (1947) mixed equal volumes of 50 per cent iso-propyl alcohol and suspension of the virus of Newcastle disease. After varying periods of exposure up to 60 minutes at 20°C portions of the mixtures were diluted with distilled water and portions of this diluted mixture inoculated into chick embryos. All of the embryos died but it must be pointed out that a 25 per cent solution is much weaker than that which gives maximum bactericidal action.

## AROMATIC ALCOHOLS

*Benzyl Alcohol*.—Of the aromatic alcohols benzyl alcohol appears to be the only one tested in recent years. It is soluble in water with difficulty, which perhaps explains its limited usage. Prombo and Tilden (1950) found 4 per cent benzyl alcohol by weight less effective than 70 per cent ethyl alcohol (either by weight or by volume) or isopropyl alcohol (99 per cent) in preventing infection in mice with *D. pneumoniae*. Benzyl alcohol, 4 per cent by weight, was slightly better than ethyl alcohol (70 per cent by weight) or isopropyl alcohol (99 per cent) in protecting mice against infection with *S. pyogenes*.

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## 15

# SURGICAL ANTISEPTICS

### INTRODUCTION

A SECTION on surgical antiseptics is indicated, partly because of the great practical importance of the subject, partly because antibacterial agents behave differently *in vitro* and on living tissues. Elsewhere in this volume separate chapters are devoted to alcohol, formaline, mercurials, bis-phenols, and quaternary ammonium compounds; here these agents will be considered together, but solely from the standpoint of their relative value *as surgical antiseptics*.

A weak link in the chain of surgical technique is the inability to sterilize skin without destroying it. The surgeon is compelled to operate with hands that cannot be fully disinfected, to make incisions through skin that cannot be made entirely germ-free. It is a matter of prime importance, therefore, to know definitely which antiseptics are most effective on skin, and how they can best be used, so that the likelihood of infection from that source may be reduced to a minimum.

It is now recognized that the action and value of antibacterial agents on skin or in wounds can be measured reliably only when these substances are tested under conditions of actual use. It is possible for a given agent to be strongly bactericidal or highly bacteriostatic in test tubes and Petri dishes and yet be relatively ineffectual in surgical practice. For three-quarters of a century, for bacteriologists and surgeons alike, this has been a controversial field of research and the subject of much unsubstantiated argument. Fortunately, in recent years, a semblance of order and agreement has begun to appear out of the confusion of claims and counter-claims.

## THE SKIN

Upon suitable magnification, the irregularly pitted, ridged and creased epidermal surface is seen to be composed of innumerable flat translucent cells which cover the underlying structures, much as a thick carpet of autumn leaves covers a lawn. These cells form the outermost layer of skin. They lie many thick, are stuck to each other and to underlying cells, and form a watertight covering for the body. The most superficial cells become desiccated, lose their attachments, and fall off. The numbers of such cells insensibly lost from the surface of the body every day is incredibly large. Bernstein (1948) counted millions of them in handwashings. Their place is taken by underlying epidermal cells which have pushed their way slowly to the surface, losing nuclei in the process, and suffering transformation from living protein-filled cells to flat masses of inert keratin. Bacteria are present on and under the superficial, loosely attached, lifeless cells, but few if any are found in or between the living cells beneath.

Looking at the magnified surface of the skin, one can also see openings of sweat ducts, and larger depressed openings through which shafts of hairs protrude. In some parts of the body sebaceous ducts also have independent openings on the surface; however, most of them empty into hair follicles below the level of the skin surface, whereupon the oily secretion works its way out alongside the hair shaft.

Sweat is normally a weak solution of sodium chloride with small amounts of urea, potassium and lactic acid, and traces of calcium, magnesium, sulfur and phosphorus. The amounts of these ingredients depend upon the profuseness of secretion. With active perspiration, the salt content rises and the nitrogen content falls. The pH may range from 4.2 to 7.5, although the ordinary variation is much narrower, from 5.02 to 5.71, according to Mosher (1933). Since normal sweat is acid and is mildly bactericidal, and the flow is continually outward, bacterial invasion of these ducts is relatively uncommon. Sweat is usually sterile as it emerges from pores onto the skin surface.

The fatty secretion of sebaceous glands is a very different substance. Its exact composition is not known, but it contains fats, soaps, cholesterol, albuminous material, epithelial cells, organic detritus and inorganic salts. Within the glands and ducts the secretion is semi-fluid, but in the open hair pits and on the exposed epidermal surface it tends to "set" into firmer consistency, coating the entire surface of the body. This coat serves a useful purpose in protecting the skin and making it water-repellent. Ordinary

washing with soap and water does not remove it fully. Sebum has very little antibacterial action; consequently, infection of hair follicles is common. Bacteria can be demonstrated occasionally in normal sebaceous ducts and glands.

Although skin is almost completely watertight, it is not equally impervious to all liquids. Many fat solvents are capable of penetrating the epidermis to greater or less degrees, and they may do harm by extracting essential lipids. Other chemicals, such as iodine and boric acid, can traverse the epidermis and enter the blood stream. Certain toxins, and probably some viruses, are also able to penetrate intact epidermis; however, bacteria and other relatively large particles are ordinarily unable to pass beneath the living surface of healthy skin.

Indeed, one of the important functions of skin is to serve as an impenetrable barrier between the bacteria-free tissues of the body and an environment which is heavily infested with microorganisms of all sorts. Any break in the skin provides a portal through which bacterial invasion of the body may occur. Since skin stands as a barricade against the germ-laden world without, it is inevitable that large numbers of bacteria of many sorts should always be collected on its exposed surface.

## THE CUTANEOUS BACTERIAL FLORA

The cutaneous flora is composed of transient and resident bacteria (Price, 1938*a*). Transients vary tremendously in number and kind. Virulent germs may be present as well as saprophytes. Test bacteria placed on skin are transients. Fortunately for our health, most of the extraneous microorganisms that get on the skin soon disappear from its surface. Some die, others fall off, or are rubbed off on clothes, or are washed off. In general, transient bacteria are more abundant on the exposed skin of the hands and face. Enormous numbers of them may be collected under the nails, on the scalp and feet, in the umbilicus and perineal area, and in any major fold or crease of the skin where microorganisms are protected and conditions are favorable for growth. Depending on the condition of the skin and the numbers of bacteria present, it takes from 7 to 8 minutes of washing with soap and water to remove all transients (contaminants) from the hands. They can be killed with relative ease by suitable antiseptics.

Residents form the stable bacterial population of the skin. They live and multiply there. Some of them die there; many are rubbed off or are washed off. Under ordinary conditions of life, increases tend to balance losses, so that on the same person the



total number of resident micro-organisms remains fairly constant. Inasmuch as the resident bacteria are firmly attached to the cutaneous surface, washing removes them slowly. They are less susceptible than transients to the action of antiseptics. Residents are composed largely of staphylococci of low pathogenicity, but some pathogenic bacteria are almost always present. The composition of the normal cutaneous flora was studied elaborately by pioneer

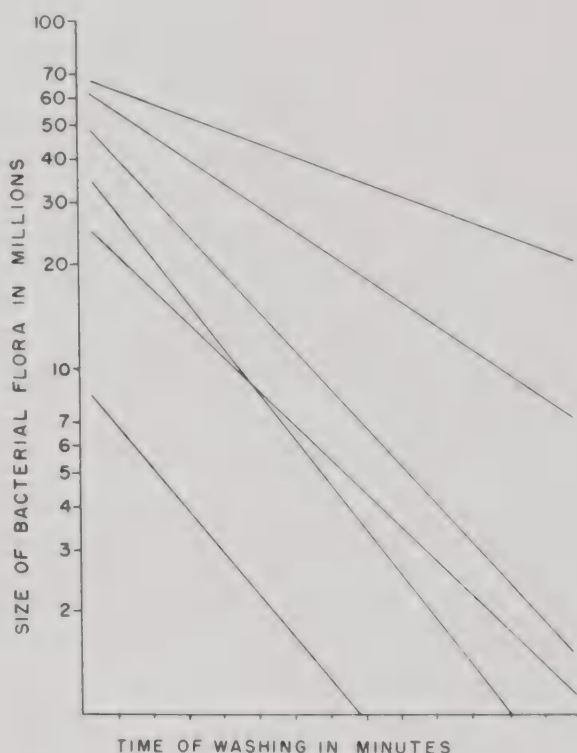


Fig. 2.—Indicating the average size of the resident flora of the hands and arms of 6 different persons, and the rate at which each flora is reduced by scrubbing in a standard manner. The transient bacterial flora, most of which comes off in the first minute of washing, is not shown. (Price, courtesy of Drug Standards.)

bacteriologists before the turn of the century (Maggiora, 1889; Preindlsberger, 1891; Welch 1892, 1895). Recent contributors to the subject include Brewer (1939), Pillsbury (1946), Lovell (1945 *a, b*), and Evans *et al.* (1950). Prolonged or frequent exposure of the skin to large numbers of bacteria may alter the ordinary composition of the resident flora and increase its potential infectiousness (Price, 1938*b*).

Some persons habitually carry a much larger flora than others (Fig. 2), but it is not possible simply by looking at skin to say whether its flora is large or small. In my laboratory, the largest

counts, surprisingly, have come from the smooth skin of young women. The rate at which the flora is reduced by scrubbing with soap and water also varies with different individuals. In general, the higher the initial flora, the slower the rate of reduction.

In addition to the resident flora just described, there is a reservoir of bacteria hidden deeply in the skin (Price, 1951*a*). The superficial resident flora comes off in washings at a regular

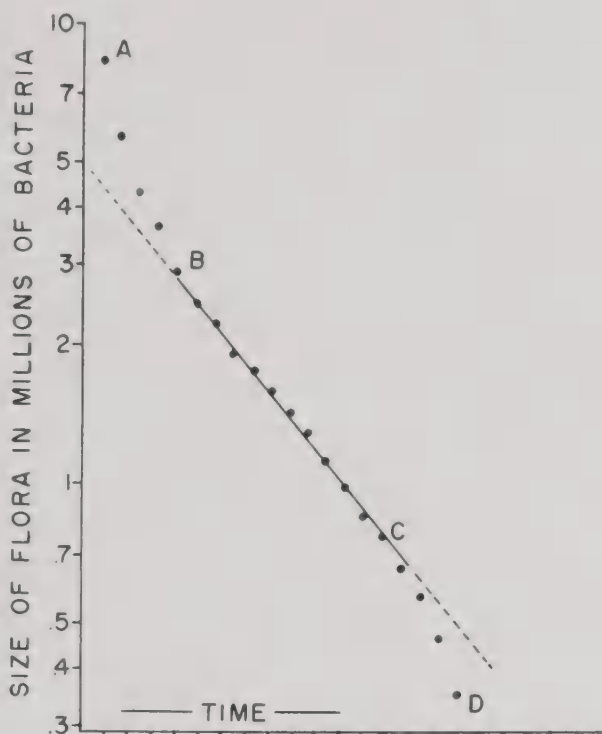


Fig. 3.—Effect of scrubbing upon the bacterial flora of the hands and arms. Results of a single test. (See text) (Price, courtesy of Drug Standards.)

rate, whereas the deep bacteria begin to appear in washings in appreciable numbers only after 15 or more minutes of scrubbing. This observation strengthens a time-honored belief that it is not possible to sterilize skin without destroying it. The deep resident flora appears to have about the same composition as the superficial resident flora; that is to say, it contains approximately the same proportion of pathogens. Little is known as yet about the size or precise location of the deep flora. It is probable that many of these bacteria come from sebaceous ducts and glands (Lovell, 1945*a, b*).

Figure 3 shows how scrubbing reduces the cutaneous flora. The curve, BC, represents the regular logarithmic rate at which the superficial resident flora is reduced by scrubbing. Deviation AB

is caused by transients which come off rapidly in the first few basins. Deviation CD is due to deep resident bacteria which appear in significantly large numbers after many minutes of scrubbing.

A spot-testing method (Price, 1951a) has been utilized to study the bacterial populations of different parts of the body surface. The distribution of bacteria on skin is found to be uneven and contiguous areas may show different sized counts, but no definite or constant patterns can be discerned. On the average, exposed skin has a resident flora approximately equal to that of skin ordinarily covered by clothing. Hands, arms, abdomen, back, thighs and (clean) feet all have counts of about the same size.

After the cutaneous bacterial flora has been reduced by scrubbing or by antiseptics, regeneration occurs, probably following a growth curve similar to that seen in cultures. Re-establishment of the usual flora requires from one to several days, depending upon the thoroughness with which the skin has been degermed (Price, 1938a, 1951a). Under rubber gloves, regeneration takes place much more rapidly.

## METHODS OF EVALUATING SURGICAL ANTISEPTICS

Historically, 3 sorts of tests have been used. First, efforts have been made to appraise antiseptics indirectly by such characteristics as deodorizing effect, surface tension, fat solubility, or penetration into skin. Unfortunately, no reliable or consistent correlation exists between any of these characteristics and ability to disinfect skin.

The second group of tests attempts to measure the inhibitory or destructive action of antiseptic agents on test bacteria *in vitro*. Robert Koch (1881) tried to find the greatest dilution of each antiseptic which would inhibit the growth of anthrax bacilli in cultures. He rated mercuric chloride first because extremely minute quantities of that substance prevented growth; and alcohol was considered almost worthless as an antiseptic because even absolute alcohol did not kill anthrax spores.

In this group also belong the phenol coefficient test, and the "spoon test" which measures the rate of killing of suspended bacteria by germicidal solutions. These tests are of interest and importance to the bacteriologist, but they are valueless to the surgeon. There is no definite correspondence between the results obtained by them and the ability of germicides to disinfect skin. Because a given chemical has a high phenol coefficient, it does not follow that a solution of that agent will disinfect the skin effectively.



In a third category are placed *in vivo* tests of antibacterial activity. The oldest and simplest of these tests consist of scraping antiseptically treated skin with a knife, culturing the scrapings, or in rubbing the skin with a moist swab and culturing the swab, or in cutting off bits of skin and culturing them in broth. Other investigators have pressed fingers on poured agar plates, or have collected sweat from disinfected hands for culture. These tests are direct approaches to the problem. They attempt to determine the effectiveness of antiseptics under conditions of use. They are of limited value, however, because they are relatively crude. They have no quantitative value. They often give falsely negative results, especially if bacteriostasis is not controlled in cultures.

In this category of *in vivo* tests also belong attempts to evaluate antiseptics by placing recognizable foreign bacteria on the skin, applying the antiseptic, and then testing to see if any of the organisms remain viable. So-called infection prevention tests are of this sort. The trouble with all such tests is that the experimental conditions are artificial. The test bacteria used are transients and not like transients. They are not bacteria that have a normal habitat on skin. They may not be the sort of micro-organisms encountered surgically. Like all transients, they are easily removed or killed compared with the resident flora. The particular organism used may not even represent the behavior of the transient flora as a whole, in that it may be easier or harder to kill than other transients. They may disappear spontaneously and rapidly from the skin. When these methods are used, bacteriostasis in cultures is often hard to control. Admittedly, these tests have a value. But the results are valid only for the particular micro-organisms used, under the conditions of the experiment. It cannot be safely assumed because a given antiseptic solution shows up well in one of these tests that it will effectively reduce the total bacterial flora of the skin.

Another *in vivo* method is the serial basin handwashing test (Price, 1938a). It is the only available test of skin antiseptics which produces faithfully the conditions of actual use, which is capable of controlling the troublesome factor of bacteriostasis, and which measures the effects of antiseptics on the cutaneous flora, quantitatively and qualitatively, with a fair degree of accuracy. It can be used to measure not only immediate effects, but also prolonged effects upon regeneration of the bacterial flora.

Off hand, one might think that the observed results of surgical antiseptics would be the final and most important test of all. Actually, the so-called clinical test of a skin antiseptic, the occurrence or non-occurrence of infection subsequent to its employment, is a crude

one. Many antiseptics have been unduly praised on that score. Subclinical infections may occur undetected in the depths of "clean" surgical incisions, and doubtless often do. Furthermore, the presence or absence of infection in operative wounds is the result of so many variable factors as to be of little significance in the evaluation of any single factor, such as the sort of skin antiseptic used.

TABLE 56.—TYPICAL SERIAL BASIN HANDWASHING TEST. EFFECT OF 1:1000 TINCTURE OF ZEPHIRAN USED FOR 2 MINUTES AT 25° C.

Basin	Size of specimen ml.	Number of colonies			Av. count per ml. bacteria	Basin totals $\times 10^3$	Cumulative totals $\times 10^3$	Calculated size of flora $\times 10^3$
		Spec. 1	Spec. 2	Spec. 3				
1	0 1	664	647	612	6,410	17,307	37,413	53,913
2	0 1	327	231	280	2,860	7,722	20,106	36,606
3	0 1	134	111	133	1,260	3,402	12,384	28,884
4	0 5	460	450	410	880	2,376	8,982	25,482
5	0 5	305	345	380	687	1,854	6,606	23,106
6	0 5	330	245	275	567	1,530	4,752	21,252
7	0 5	235	360	310	603	1,629	3,222	19,722
8	0 5	345	275	265	590	1,593	1,593	18,093
								16,500*
2-minute wash without friction in Zephiran, followed by brief rinse in sterile water								
9	1 0	167	152	156	158	427	2,036	3,636
10	1 0	119	102	93	105	283	1,608	3,208
11	1 0	71	71	93	78	211	1,326	2,936
12	1 0	86	92	90	89	241	1,114	2,714
13	1 0	110	123	96	110	296	873	2,473
14	1 0	53	58	66	59	159	577	2,177
15	1 0	46	55	58	53	143	418	2,018
16	1 0	55	57	60	57	155	275	1,875
17	1 0	44	45	44	44	120	120	1,720
								1,600*

Each basin, containing 2700 ml. sterile water, is scrubbed in uniformly for 60 seconds.

\* Calculated number of bacteria left on the hands and arms after each series of washings

Because the serial basin handwashing test has become a standard procedure, a brief description of the method is appropriate. (1) The hands and arms are scrubbed with brush and Ivory soap in a series of 8 or more basins of sterile water. Sixty seconds are spent in each basin. Exactly the same method of soaping, brushing, and rinsing is repeated each time. (2) The hands and arms are permitted to dry by evaporation. (3) They are then washed in the antiseptic to be tested for a measured length of time. Factors of temperature and friction are controlled. At the end of the test period, the excess antiseptic is immediately rinsed off with sterile water. (4) The hands and arms are at once transferred to a basin containing an appropriate solution designed to neutralize bacteriologically any remaining antiseptic on the skin. This is an important step (see Klarmann, 1950); otherwise misleading bacteriostasis may occur in subsequent cultures. For example, acids should be followed by an alkali, mercurials by an alkaline sulfide or some other reducing agent, iodine by sodium thiosulfate, Zephiran by

soap. Alcohol has no specific antidote, but it can be rapidly diluted to a degree where it loses all antibacterial action. (5) Finally, the hands and arms are scrubbed in the same uniform manner in a

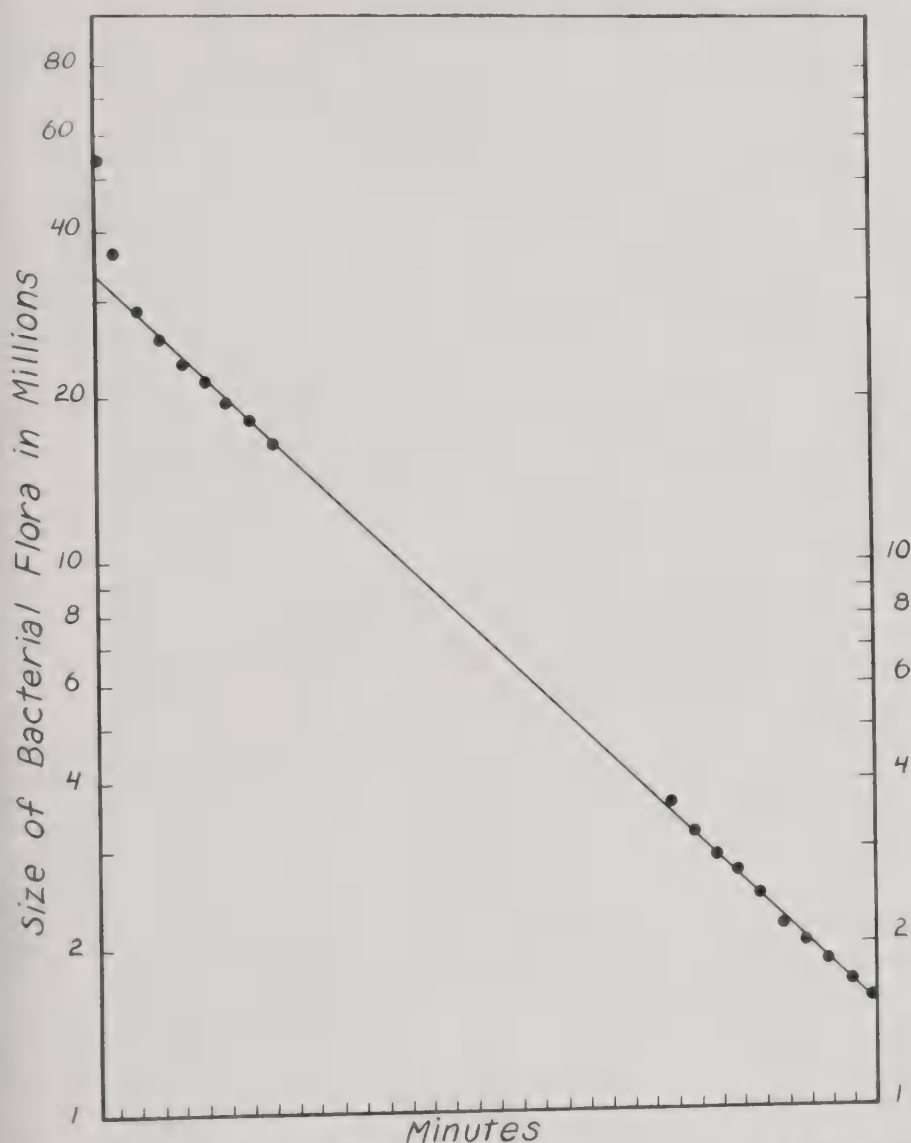


Fig. 4.—Curve of Table 56. The sloping line represents the rate at which this subject habitually reduced the resident cutaneous flora by standardized scrubbing; the points represent the “actual totals” of the two series of washings, shown in the last column of Table 56. It is clear that the degerming effect of washing the hands and arms for 2 minutes in tincture of Zephiran was equivalent to many minutes of scrubbing.

second series of 8 or more basins, using a fresh brush and cake of soap. (6) Samples of all washings are taken without delay for pour-plate cultures. Infusion agar is used. Incubation is for 48 hours or longer. (7) Results are tabulated, as in Table 56. (8) From these data, curves are plotted (Fig. 4), and the size of the



flora before and after the use of the antiseptic is computed. The 2 curves, which represent the first and second series of washings, are calculated and plotted independently of each other. These calculations may be made by mathematical means (Bernstein, 1942, 1948), or more simply by means of a graphic method (Price, 1938a).

Many investigators have employed modifications and simplifications of the test just described. Most of them err, however, in placing undue emphasis on bacterial counts of single washings. It has become popular to compare the size of such counts immediately before and after application of a given antiseptic. Trustworthy conclusions can scarcely be drawn from such comparisons. All too often in these tests bacteriostasis has not been adequately controlled. Besides, the important problem is not the number of live bacteria in the washings, but the number of potentially dangerous bacteria left on the skin. The counts of washings are meaningful, and can be trusted to evaluate antiseptics, only when they are used to calculate the size of the cutaneous flora, and the extent to which that flora is reduced by antiseptic agents.

### ETHYL ALCOHOL

Early in the modern surgical era, alcohol was used on the skin primarily as a detergent; only later did its antiseptic properties come to be appreciated. Epstein (1897) held that 50 per cent by volume was the most effective antiseptic concentration—an error which persists in some textbooks to this day. Harrington (1903), experimenting with threads impregnated with test bacteria, concluded that 60 to 70 per cent (by volume) was best for dry organisms, but that stronger solutions were equally efficacious against wet bacteria. Beyer (1911) was the first to point out the importance of preparing solutions of alcohol by weight rather than by volume. He found exactly 70 per cent by weight by far the most germicidal concentration *in vitro*. In recent times, alcohol has become the most widely used and trusted of all skin antiseptics (Price, 1950c).

The mechanism of action of alcohol on the cutaneous bacterial flora is not fully known. Apparently, it is not due to fat solvent properties (Price, 1939a), inasmuch as 70 per cent alcohol is a poor fat solvent. Bernstein (1948) has shown that it is not simply a matter of “hardening” of the epidermis with “fixation” of bacteria on its surface, as some have suggested. Deceptive bacteriostasis in cultures can also be eliminated as an explanation. The degerming action on skin appears rather to be true bactericidal effect *in situ*; hence the action of alcohol can be enhanced by raising the tempera-

ture of the antiseptic, or by using gauze or washcloth friction to ensure better contact between the alcohol and the local bacterial flora.

TABLE 57.—GERMICIDAL EFFECT OF ETHYL ALCOHOL ON *M. pyogenes* var. *albus*\*  
(PRICE, COURTESY OF ARCH. SURG.)

Duration of contact between germicide and bacteria	Concentration of alcohol, per cent by weight									
	10	20	30	40	50	60	70	80	90	99
0 (control)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1 second	108.3	104.5	80.2	71.4	68.8	59.5	27.1	29.9	68.8	61.9
10 seconds	86.7	94.6	65.8	77.6	41.2	34.9	26.6	26.6	37.3	60.7
30 seconds	106.5	92.7	50.2	47.2	35.7	18.4	21.2	34.1	48.2	48.7
1 minute	127.5	91.1	49.8	35.5	22.7	9.3	18.6	14.9	54.4	46.8
5 minutes	108.4	89.9	42.2	14.1	15.5	12.2	21.7	24.4	41.9	26.2
10 minutes	90.4	73.6	24.6	14.7	9.9	13.5	23.4	20.1	46.0	41.8

\* The numbers in the table indicate the per cent of test organisms surviving after contact with alcohol.

The rate at which different strengths of ethyl alcohol kill aqueous suspension of *Micrococcus pyogenes* var. *albus* (the predominating micro-organism of normal skin) *in vitro* at 25° C is clearly shown in Table 57. Similar studies made with *Micrococcus pyogenes* var. *aureus* and *Escherichia coli* have been reported (Price, 1950a).

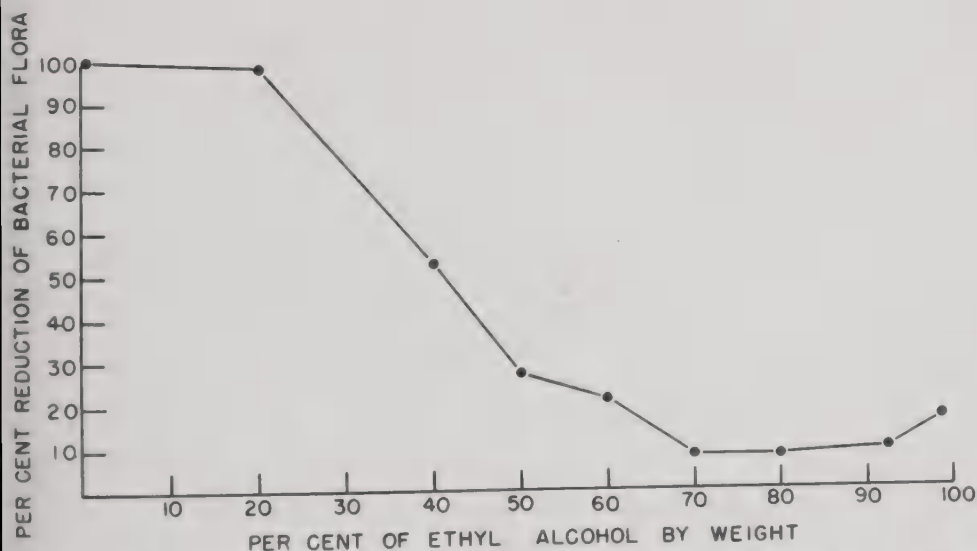


Fig. 5.—Reduction of the cutaneous bacterial flora by various strengths of ethyl alcohol. Average of over 60 tests on several individuals. Alcohol applied at 25° C without friction.

The value of alcohol as a skin antiseptic is best measured, however, by means of the serial basin test just described. Results of more than 60 such tests made in the author's laboratory on four different subjects are summarized in Figure 5. This curve indicates

the ability of alcoholic solutions of various concentrations to reduce the flora of the hands and arms when applied for 120 seconds without friction at 25° C. In each instance, the bacterial population just before application of alcohol was considered as 100 per cent, and the total calculated number of viable bacteria remaining on the skin immediately after the alcohol was given a proportional percentage value.

It is clear from these studies that 20 per cent alcohol has almost no degerming effect, but that solutions between 20 and 70 per cent have progressively more powerful antiseptic action. Strengths between 70 and 92.3 per cent (equivalent of 95 per cent by volume) all have about the same antiseptic action. These solutions destroy the transient flora quickly, and they are capable on the average of reducing the resident flora to about 8 per cent of its initial size in 2 minutes. Additional studies show that if the alcohol is applied with gauze or washcloth friction, this antiseptic action is approximately doubled.

In this connection, it is significant that of 11 persons tested, none had a cutaneous flora that was resistant to this relatively strong and rapid action of alcohol (Price, 1951*a*). That is not true of all antiseptics, as will be shown later.

For routine surgical use, 70 per cent alcohol by weight is recommended by the author for several reasons: it is strongly germicidal; it is somewhat less expensive, volume for volume, than the more concentrated preparations; it wets the skin well, spreads smoothly, and evaporates slowly, thus giving the alcohol ample opportunity to act upon the bacterial flora; it does not injure keratin or extract lipids of the epidermis, and in consequence it is almost perfectly innocuous on healthy skin. Only rarely have I encountered irritation of skin by this solution, and in those instances the fault seemed to lie with denaturing substances in the alcohol, rather than with the alcohol itself.

## PROPYL ALCOHOL

An extensive study of propyl alcohol made in my laboratory (unpublished) is the basis for the following comment:

Both normal propyl alcohol and isopropyl alcohol, when subjected to the serial basin handwashing test, are found to be slightly superior to ethyl alcohol in skin antiseptic effect. The degerming action is optimal when solutions 70 per cent (by weight) and stronger are used. Since isopropyl alcohol is non-potable, it may be obtained readily, in unadulterated form, and without the annoying restrictions and taxes ordinarily associated with ethyl alcohol.



On the other hand, the propyl alcohols are more efficient fat solvents than ethyl alcohol, and consequently they are somewhat harder on the skin. Isopropyl alcohol might well be substituted for ethyl alcohol to prepare the skin of the field of operation, but it is not recommended for repeated washing of operators' hands.

## MERCURIALS

Correct evaluation of mercurials as skin antiseptics is peculiarly difficult. Many investigators working in this field have been led astray; consequently, no group of antiseptics has in its day received more enthusiastic acclaim, but in the long run has proved to be more disappointing.

Mercuric chloride was highly recommended by Koch (1881), Laplace (1887), and other early investigators. Koch placed mercuric chloride at the top of his list of antiseptics, and it soon came to be widely used. Its popularity gradually diminished, however, after Geppert (1889, 1891) demonstrated that its action is more bacteriostatic than bactericidal; and that if "sublimated" test bacteria are treated with ammonium sulfide so as to precipitate the mercury in the form of an inert sulfide, the organisms will grow readily in cultures. Two or 3 decades ago, a number of organic mercury compounds were introduced and popularized as skin antiseptics. Unfortunately, these also were tested initially without the corrective of suitable antagonists, with the result that an exaggerated opinion of their antiseptic power became widespread.

Mercurials are highly bacteriostatic. In making tests, small quantities carried over into cultures by loops or swabs or scrapings may inhibit growth. More than that, both inorganic and organic mercurial compounds combine with bacterial cells in some sort of union or complex that is not broken by simple washing in water or by excessive dilution. As a result, deceptive degrees of bacteriostatic or slow bactericidal effect almost invariably occur in cultures unless suitable antidotes are also utilized. Furthermore, there is evidence that mercurials react with the epidermis to form a "film" which covers over the underlying bacteria without injuring them or preventing their multiplication. This characteristic behavior also contributes to the illusion of a sterile cutaneous surface. To complicate the matter further, alkaline sulfides and other reducing agents applied to skin seem to *increase* the size of the bacterial flora. This phenomenon has not been satisfactorily explained. These problems have been discussed in detail by Price (1939*b*, 1951*b*), Cromwell and Leffler (1942), and others.

Taking all of these disturbing factors into consideration, the author has made an effort to evaluate the skin antiseptic action of some of the more familiar mercurial preparations on the basis of the serial basin handwashing test. Figure 6 shows the results

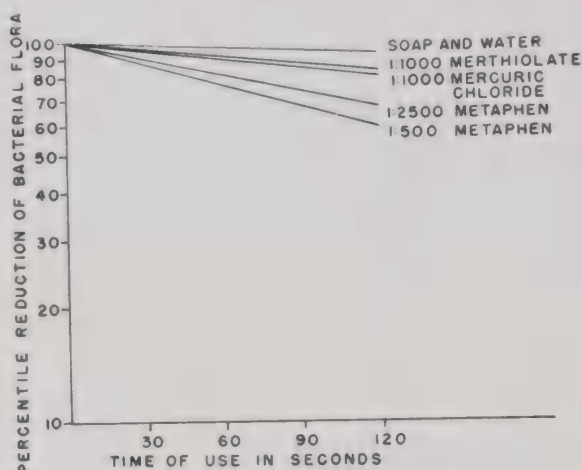


Fig. 6.—Comparative effects of aqueous solutions of selected mercurials upon the bacterial flora of the hands and arms. (Price, courtesy of Drug Standards.)

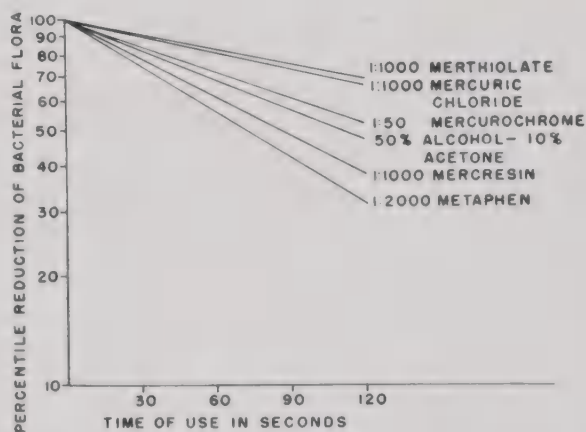


Fig. 7.—Effects of alcohol-acetone solutions of mercurials upon the bacterial flora of the hands and arms. All solutions used at 25° C without friction. (Price, courtesy of Drug Standards.)

obtained with aqueous solutions. In this chart, for the sake of simplicity and ease of comparison, the effects of scrubbing are not indicated, but only the degerming effects of the antiseptics. Thus, 1:1000 merthiolate, used for 2 minutes, reduced the bacterial flora to about 82 per cent of its original size. The steeper the slope of these curves, the greater the antiseptic action. All of these antisep-

tics were applied with light gauze friction, much as one paints a patient's skin in preparation for operation. It can be seen that the best of these solutions has reduced the flora by less than one-half in 2 minutes.

For comparison, I have included the effect of washing in a similar manner with soap and water. It is often stated that soap and water are as effective antiseptic agents for the skin as anything else. Obviously that is not correct. Soap removes grease, dirt, and loosely attached contaminating bacteria, and for that reason its use is important, but it does not reduce the resident bacterial flora efficiently.

Alcoholic solutions of these same mercurials (Fig. 7) were more effective than the corresponding aqueous solutions, but the difference was due mainly to the antiseptic action of the alcohol-acetone solvent. Indeed, the antiseptic action would have been greater in some instances if the solvent had been used without the mercurial.

## IODINE

Iodine has a long and honorable tradition. For half a century it was universally used and trusted to prepare the skin for major and minor operations. Few investigators, including those who were careful to employ effective antidotes, failed to rate it highly as a skin antiseptic. In recent times, however, it has fallen into disuse because of a general feeling that it is apt to be irritating to skin and other tissues, and that milder antiseptics will do as well.

When iodine was tested by the serial basin method (Fig. 8), superior results were obtained (Price, 1938*b*, 1951*b*). Whereas the skin antiseptic action of alcohol appeared to be decreased by addition of mercurials (Fig. 7), its effect was increased by the addition of iodine.

One or 2 per cent iodine dissolved in 70 per cent alcohol is an excellent skin antiseptic. It spreads evenly, dries slowly, and evaporation does not leave a rim of concentrated iodine to burn the skin. Used judiciously it does not often cause irritation. The old U.S.P. tincture of iodine (7 per cent) is even more potent bacteriologically, but it may be painful and even damaging to skin. "Half-strength" tincture of iodine (3.5 per cent) is unsatisfactory because it dries too quickly and unevenly, leaving streaks or islands of concentrated iodine with resultant burns. Aqueous solutions of iodine probably should not be used on large areas of skin. Since they are more highly ionized than alcoholic solutions, they are dangerous and may cause severe burns. The author has had a case of iodism



occur in his laboratory from washing the hands and arms for 30 seconds in Lugol's solution.

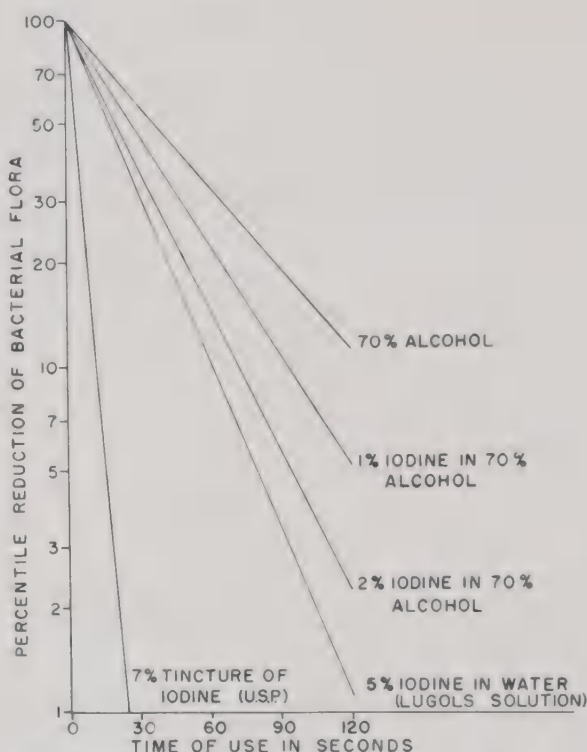


Fig. 8.—Effects of 70 per cent alcohol (by weight) and various solutions of iodine upon the bacterial flora of the hands and arms. (Price, courtesy of Drug Standards.)

### BENZALKONIUM CHLORIDE (ZEPHIRAN)

This quaternary ammonium compound, introduced by Domagk in 1935, has become a popular skin antiseptic in operating rooms. Much of the work done to evaluate it has been vitiated, however, by failure to recognize the bacteriostatic influence of extremely small amounts of the agent in cultures, especially for staphylococci, and by failure to appreciate fully, when Zephiran is used, the neutralizing effects of minute traces of soap that are nearly always present on skin.

Zephiran has been restudied recently (Price, 1950b) with methods which are better able to control those disturbing factors. *In vitro*, Zephiran is a powerful, rapidly acting germicide against test bacteria, but on skin, under conditions of ordinary use, its antiseptic action is not as great as many investigators have believed. Figure 9 summarizes the results of a large number of serial basin tests. Evidently, the effectiveness of the agent can be enhanced if

it is employed with a clear understanding of its peculiarities and behavior.

Hands and arms that have been scrubbed in the usual manner preoperatively need to be very thoroughly rinsed with water (for 1 minute or more) in order to remove the soap which clings so tenaciously to skin and tends to neutralize the antiseptic action of Zephiran.

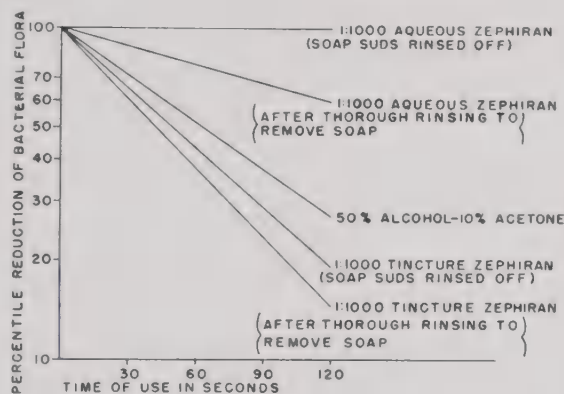


Fig. 9.—Effects of Zephiran upon the cutaneous bacterial flora. The importance of thorough removal of soap from the skin before applying Zephiran is clearly demonstrated. (Price, courtesy of Drug Standards.)

Since solutions of alcohol are better soap solvents than water, it is recommended that the site of operation be washed alternately several times with 70 per cent alcohol and 1:1000 tincture of Zephiran. It should be pointed out in this connection that, bacteriologically speaking, hexachlorophene soap and tincture of Zephiran tend to neutralize each other.

## HEXACHLOROPHENE (G-11)

Hexachlorophene, a bis-phenol, is one of the few known antiseptics that does not lose most of its antibacterial potency in the presence of soap. Consequently, hexachlorophene has been combined with soap and other detergents, and has been recommended for surgical preparation of hands and the field of operation.

Highly favorable reports have been published by Traub *et al.* (1944), Udinski (1945), Seastone (1947) and others. Walter (1948) and associates proposed the use of a synthetic detergent vehicle (pHisoderm) with 3 per cent hexachlorophene, which, it is claimed, is even more effective than the soap preparations. It has been asserted that persons who operate regularly no longer need to scrub in the old-fashioned manner, nor soak their hands in irritating solutions; instead, it is necessary only to lather the hands and arms

for 2 or 3 minutes with hexachlorophene soap. It is alleged that washing with G-11 soap once a day will not only reduce the cutaneous flora to a very low level, but keep it persistently low. These glowing recommendations, together with the desire of many surgeons for a quick easy method of preparing for operation, have resulted in an extraordinarily rapid adoption of hexachlorophene preparations in the hospitals of this country. In a great many operating rooms at the present time, hexachlorophene soap and pHisoderm have replaced the conventional scrub and alcohol wash. And in some hospitals, the field of operation is prepared by a brief wash with hexachlorophene soap and water, followed by application of tincture of Zephiran.

It is only natural that the initial enthusiastic reports about hexachlorophene should be followed by more critical re-evaluation. Price and Bonnett (1948), Blank and Coolidge (1950), Blank *et al.* (1950) and Price (1951a) conclude that single washes with G-11 soap are not very effective in reducing the cutaneous flora.

Correct evaluation of the antibacterial power of hexachlorophene is fraught with peculiar difficulties because of its insolubility in water, the bacteriostatic effects of even minute traces of the substance in cultures, and the lack of an effective neutralizing agent. Extreme care must be taken, therefore, in making tests and in interpreting the results.

The author, after two years of intensive experimental and clinical study of the substance, came to the following conclusions (Price, 1951a):

"Quantitative and qualitative tests of skin disinfection, using the serial basin handwashing method and a new 'spot testing' technic, with control of bacteriostatic effects in cultures, demonstrate that the presently popular brief period of handwashing with soap or a synthetic detergent containing hexachlorophene (G-11) is not as efficacious as has been so enthusiastically claimed.

"Hexachlorophene does not disinfect the skin quickly, as alcohol does, but much more slowly . . . This slow degerming action is attributed to a film of the agent left on the hands after washing with the medicated soap. In order to achieve this desired effect, it is necessary to use G-11 soap exclusively and frequently (*i.e.*, many times a day) . . .

"It appears that the bacterial populations of the hands of different people vary in susceptibility to the agent. The fact that some individuals harbor a bacterial flora that is resistant to the action of hexachlorophene injects a disturbing element of uncertainty into its exclusive use in preparing the hands for operation. Even in those persons whose cutaneous bacterial flora has been



shown by appropriate tests to be sensitive to hexachlorophene, an occasional brief wash with G-11 soap cannot be relied on to keep the hands relatively free from infectious germs (as has been alleged), either under rubber gloves or in ordinary conditions of life.

"Comparative studies show that the conventional scrub followed by a three-minute wash in 70 per cent alcohol is much more effective bacteriologically, and more consistently so, than a brief lathering with G-11 soap or pHisoderm."

## DETERGENTS

A number of surface active or wetting agents, both anionic and cationic, obtained from various chemical and drug firms, have been tested by means of the serial basin technic for their degerming effects on the skin (Price, 1951*b*). None of these products *per se* reduced the cutaneous flora any more rapidly than ordinary soap.

Ether applied to skin with a gauze sponge has almost no effect on the size of the resident bacterial flora, but when the hands and arms are submerged in ether for one minute, the flora is reduced considerably. This latter procedure is not painful, but it is followed by uncomfortable dryness and exfoliation of the skin.

Acetone is found to be a less effective antiseptic than alcohol. It is somewhat injurious to skin.

Chloroform and zylene have an antiseptic action comparable to that of alcohol, but both substances are so irritating and harmful that they should not be used on skin either as detergents or as antiseptics.

## MISCELLANEOUS AGENTS

The old-fashioned lime-and-soda method of preparing the hands for operation is a rather effective one from a bacteriological standpoint. The degerming action is due partly to the soda, partly to the hypochlorite and nascent chlorine. It has never been a popular method, however, because it is troublesome to use and the odor is disagreeable.

Methyl alcohol is slightly inferior to both ethyl and isopropyl alcohols.

Saponated cresol solution (Lysol) is strongly bactericidal when set against organisms *in vitro*, but in concentrations that are not injurious to the hands, it is ineffective as a skin antiseptic (Price, 1938*b*, 1951*b*).

Ceepryn and Phemerol, quaternary ammonium compounds, cause moderate reductions in the cutaneous flora, especially when used in alcohol solutions, but they are inferior to Zephiran.

Dettol, a halogen derivative of xylenol, recommended by Colebrook and Maxted (1933), apparently is still used to some extent in Britain. The serial basin test shows that it has relatively slight effect upon the resident bacterial flora of the skin.

## MUCOUS MEMBRANES

Not much is known about the bacteriology of mucous membranes, and still less about the effective use of antiseptics on those surfaces.

The mucous membranes of the eye, nose and urethra normally harbor relatively few bacteria as compared with the nearby skin. This is probably due largely to the smooth moist surfaces which do not offer a ready foothold for contaminating microbes, and to the flow of secretions which carry the bacteria away. Moreover, these fluids all have slight but perhaps significant antibacterial properties. Even so, it is remarkable that these soft delicate membranes, only a few cells thick, provide so effective a barrier against bacterial invasion. Some virulent bacteria and viruses appear to be able to penetrate the intact mucosa, but as a rule inflammation and infection follow injury of the epithelial surface by trauma or chemical irritation. When infection becomes established, great numbers of bacteria may be present on the mucosal surfaces and in their secretions.

In operating on these areas, surgeons customarily and properly are concerned primarily with antisepsis of the exposed skin with its relatively large bacterial flora, and less with the possibility of wound infection from the mucosa itself. Some surgeons use antiseptic preparations which are so mild that they will not irritate mucous membranes. At best, these solutions are feeble, slowly acting antiseptics. If one may judge by their action on skin, they do not have much bacteriological effect other than mechanical cleansing of the mucosal surface.

A different problem is presented by the mouth and pharynx. Here the microbial flora is much more varied and vastly greater. Many sorts of micro-organisms, aerobic and anaerobic, may be present (Dubos, 1952). Unpublished studies by the author indicate that even "clean" healthy mouths may contain a bacterial flora more than a thousand times that of the hands and arms. Unbrushed teeth, caries, gingivitis, pyorrhea, inflammations and ulcerations may add materially to the flora. The neglected mouth of an unconscious patient may contain incredibly large numbers of bacteria. Mechanical cleansing with toothbrush and mouthwashes remove many microorganisms, but countless numbers remain. The neces-

ity of operating in the presence of huge numbers of microbes, many of them pathogenic, poses a difficult problem for the surgeon. Conventional antiseptic mouthwashes are not of much value here; instead, the surgeon has to depend largely on natural body defenses, abundant blood supply of the tissues, open wounds, and chemotherapy. An effective mouth antiseptic would make surgery of that region safer and there would be less postoperative inflammation and pain.

The mucosal surfaces of the anus and of the vagina and female external genitalia also harbor a great many bacteria. As would be expected, organisms of the colon group usually predominate. Here again, gentle mechanical cleansing is the most important single step in preparing the field for operation. Antiseptics which are harmless to the mucous membranes do not contribute much to disinfection of the parts. Frequent washing with a hexachlorophene preparation for 4 or more days is probably a good method of preparing the area for operation (Walter, 1948).

The esophagus is almost always highly contaminated, but the normal empty stomach is relatively free from bacteria, due to the bactericidal action of its secretions. It is a curious fact that the grossly contaminated intestinal tract, which is so inaccessible, and is lined with countless intervillous spaces and glands in which bacteria may hide, is the one mucosal area of the body in which antiseptic efforts have been most successful. This has been accomplished by the ingestion of poorly absorbed sulfonamides and antibiotics which remain in the intestinal lumen and act slowly on its bacterial flora.

## WOUNDS

The bacteriology of wounds has received an enormous amount of study. The search for some antiseptic which will destroy contaminating bacteria in wounds without injuring the body tissues is a very old one. No such ideal antiseptic has yet been found. The best that can be done at the present time is to disinfect the surrounding skin, cleanse the wound surgically, ensure good circulation, dress the wound infrequently, put the part at rest, use specific chemotherapy, and rely upon the natural defenses and healing processes of the body, aided later perhaps by secondary suture or skin grafting. Only rarely is the use of antiseptics in wounds indicated. A notable exception is the employment of activated zinc peroxide in the treatment of micro-aerophilic and anaerobic infections of the skin and subcutaneous tissues.



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## 16

# ANTISEPTIC OINTMENTS

## INTRODUCTION

ROBERT KOCH (1881) established the fact that 5 per cent solutions of phenol in oil or alcohol did not exhibit antiseptic properties such as were observed for aqueous solutions of phenol. Although Koch worked with oil solutions, it was realized that similar effects might occur with semi-solid fatty ointment bases. Since that time, numerous investigations have been carried out to determine the effects of various ointment bases on the efficacy of antiseptics incorporated in the bases.

## PHENOL OINTMENTS

For many years phenol has been considered a standard for the evaluation of antiseptics. Furthermore, ointments and other pharmaceutical preparations of phenol have been used extensively in medical practice. For this reason the results of research on the antiseptic value of phenol ointments are of particular interest.

Koch (1881) stated that phenol when dissolved in oil or alcohol does not exhibit any antiseptic properties. In Koch's experiments, silk fibers impregnated with spores of *Bacillus anthracis* were immersed in various phenol solutions in tightly stoppered test tubes. After definite periods the fibers were taken out with the aid of a platinum loop and transferred into gelatin, blood serum or bouillon broth, and the growth and development of the organism observed. Aqueous solutions of phenol of 1 and 2 per cent strength were ineffective, as growth was observed even after immersion for 15 days and 7 days, respectively. Growth was completely inhibited by 3 per cent phenol in 7 days, by 4 per cent in 3 days, and by 5 per cent in 2 days. In similar tests using the bacilli themselves instead of spores



o growth was observed even after immersion in 1 per cent aqueous phenol solutions for only 2 minutes, while the controls showed abundant colonies. Koch next tried 5 per cent solutions of phenol in oil and in alcohol. The inefficiency of such oily or alcoholic solutions was shown by the fact that the spores were not killed in 110 days, and with the bacilli the growth was completely inhibited only after 6 days.

The behavior of phenol in oil was explained by Wolffhügel and von Knorre (1881) on the basis of the difference in diffusibility of oily and aqueous solutions. A 5 per cent solution of phenol in oil was brought into direct contact with water, and the amount of phenol which diffused into the water was determined by the Landolt and Koppenschaar method in which phenol is converted to tribromophenol and the excess bromine is titrated. The proportion of phenol which diffused into water from oil was negligible compared with that which went over from an aqueous solution into oil.

Gottstein (1889) experimented with 5 per cent of phenol in lanolin base. Small amounts of the ointment were inoculated with *Bacillus marcescens* and *Pseudomonas fluorescens* and a drop of the mixture transferred into nutrient gelatin. Growth of the organisms was not inhibited by the phenol ointment. On the other hand, mercury bichloride when incorporated in lanolin showed the same antiseptic qualities as it did in aqueous solution. Hence Gottstein concluded that oil-soluble antiseptics when incorporated in an ointment base do not possess any antiseptic properties, but that water-soluble antiseptics do not lose their antiseptic properties when incorporated in oily bases.

Experiments were carried out by Breslauer (1895) with the object of determining to what extent antiseptic substances exert their germicidal action when incorporated in ointments and whether the antiseptic effect can be influenced by varying the bases. Cultures of the test organisms were grown on agar for 2 days at 22° C. The colonies were then transferred into 2 ml of bouillon, vigorously shaken and filtered through glass wool. A number of glass platelets were then introduced into the bouillon suspension, removed after a short time and dried on a Petri dish. The dried platelets were next placed in the ointment to be tested, and after a definite period of time they were taken out, washed with ether to remove the ointment, transferred into sterile bouillon tubes, and observed for growth of the organism. Ointments containing 5 per cent of phenol in various bases were tested against *S. marcescens*. The time after which growth was inhibited was as follows: oil, 3 days; petrolatum, 90 minutes; fat, 45 minutes; lanolin (anhydrous), 20 minutes; lanolin, 15 minutes; cold cream, 5 minutes. Similar experiments were car-

ried out with *Micrococcus pyogenes* var. *aureus*. Breslauer concluded that lanolin and cold cream are the best bases for antiseptics.

During World War I, Cheyne (1915) worked with various antiseptic ointments with the hope of finding one that would prevent sepsis in wounds. Ointments spread on cover glasses were placed in Petri dishes under agar slabs, the ointment being next to the agar. The upper surface of the slab was inoculated by brushing on a thin emulsion of the bacteria employed, such as *M. pyogenes* var. *aureus*. The plates were incubated at body temperature and observations of the bacterial growth were made from time to time. Using 20 per cent phenol in 5 different ointment bases, best results were obtained with lanolin and poorest results with a hydrocarbon base. The addition of wax to the lanolin decreased the antiseptic effect by about one-quarter. Using Cheyne's technique, Keilty and Packer (1915) rated ointments containing 10 to 20 per cent of phenol as markedly active.

An improved method involving the spreading of the ointment on an inoculated agar medium was devised by Reddish (1927) and adopted by the United States Food and Drug Administration (Ruehle and Brewer, 1931). In this test, which is called the agar-plate method, a culture of *M. pyogenes*, var. *aureus* of normal resistance is thoroughly mixed with melted nutrient agar, which is then poured into a sterile Petri dish and allowed to cool to room temperature. As soon as this inoculated agar has hardened, the ointment is spread over a small portion of the surface with a sterile glass rod and the plates are incubated for 24 to 48 hours. If the ointment has an antiseptic effect there is a clear zone around the ointment.

In a study of the antiseptic action of various U.S.P. and N.F. ointments, Reddish and Wales (1929) found that Ointment of Phenol, U.S.P. X, did not show antiseptic properties by this test; this ointment consists of phenol, 2 g; yellow wax, 5 g; and petrolatum, 93 g.

In view of the lack of antiseptic effect shown by the official phenol ointment, an extensive study was made by Husa and Radin (1932) to determine the effect of variations in the base on the antiseptic properties of phenol ointments. In evaluating the ointments, the Reddish (1927) method was used, with some modifications. One modification consisted in using a weighed quantity of ointment in the test and smearing it over a definite area, since it had been found that variations in the quantity of ointment used in the test had a small but definite effect on the width of the zones. Hence, for standardization of results, 0.5 g of the ointment was smeared over a circular area of 4 cm<sup>2</sup>, the area being outlined on

the under side of each dish with a colored wax pencil. Tests were conducted to determine what percentage of phenol incorporated in petrolatum was necessary to show antiseptic properties. It was found that when the proportion of phenol was 5 per cent or less there was no zone. Ten per cent of phenol gave a 4-mm zone and higher percentages of phenol gave larger zones. However, 5 per cent of phenol in a base composed of 23 to 24 per cent of anhydrous lanolin and 76 to 77 per cent of petrolatum had such a pronounced antiseptic effect that the entire plate was clear. With a 2 per cent concentration of phenol, 5-mm zones were obtained with bases containing 25 to 25.5 per cent anhydrous lanolin and 74.5 to 75 per cent petrolatum. Since the official ointment contained 5 per cent of yellow wax, tests were made with ointments containing 2 per cent of phenol and 5 per cent of yellow wax with the remainder consisting of various proportions of anhydrous lanolin and petrolatum. No zone was obtained with any of these ointments, thus indicating that yellow wax reduces the antiseptic properties of these ointments to zero on the basis of the agar-plate method.

Bryan (1936) verified the results of Husa and Radin (1932). His results showed no zone for 5 per cent phenol in petrolatum but a zone was obtained with 5 per cent phenol in a mixture of 75 per cent anhydrous lanolin. He likewise found that yellow wax reduced the antiseptic properties of phenol ointments to zero.

The antiseptic effect of anhydrous lanolin-petrolatum ointments containing only 2 per cent of phenol was apparently of a marginal nature since some laboratories obtained negative or variable results. For example, Gershenfeld and Miller (1933*a*) obtained small zones in 18 out of 40 tests. These variable results seemed to indicate that slight differences in conditions and technique might have considerable effect on the width of the zones. Further work (Husa and Radin, 1933) indicated that with other conditions equal, the quantity of agar medium employed was the decisive factor. Using 10 ml of agar medium, zones of 5 mm were obtained with an ointment consisting of 2 per cent phenol in a mixture of 25 per cent anhydrous lanolin and 75 per cent petrolatum. However, when using 20 ml of agar medium, there were no zones of inhibition. Hence it appears that the F.D.A. method (Ruehle and Brewer, 1931) could be further standardized by specifying a definite number of ml of agar rather than allowing the use of 15 to 20 ml. It would also be an improvement to specify that a definite weight of ointment be applied to a definite area of the agar medium.

It was shown by Craw and Lee (1938) that 2 per cent of phenol in benzoinated lard is an effective antiseptic as indicated by 8-mm zones obtained in the agar-plate method. Gershenfeld and Miller



(1933b) found that vanishing cream bases containing 2 per cent phenol gave distinct zones showing bactericidal effect with five bases while three bases produced only a bacteriostatic action.

In spite of the definite evidence that U.S.P. X Ointment of Phenol had no antiseptic effect, the same formula was retained for the U.S.P. XI. The reasons given for this action were that medical opinion led to the conclusion that Ointment of Phenol was intended as a bland dressing having a local anesthetic effect of phenol and it was feared that if the phenol left the ointment base and entered the tissues it might cause gangrene. However, the research on phenol ointments was not fruitless since it increased the general knowledge of antiseptic ointments and brought out facts and principles that were later applied to other ointments.

### FACTORS AFFECTING ANTISEPTIC ACTION

*The theoretical aspects* of the action of antiseptic ointments appear to be as follows. In order for an ointment to exert an antiseptic effect it is necessary that the antiseptic substance leave the base and enter the underlying tissues which may harbor pathogenic bacteria. In the case where the ointment and the culture medium or tissues are immiscible, the distribution of the antiseptic substance between the ointment base and the culture medium or tissues at final equilibrium would be in accord with the Nernst partition law, which states that if solvents are immiscible, there is a constant ratio of concentration of solute in the two solvents provided the molecular weight of the solute is the same in both solvents. However, in the agar-plate method as well as in clinical use of ointments, all of the antiseptic substance is present at first in the ointment phase and the effect of the ointment is dependent on the rate at which the antiseptic substance is able to diffuse into the tissues or agar medium. In order for the antiseptic effect to be apparent in the agar-plate test, it is necessary that the antiseptic reach a sufficient concentration in the agar to prevent growth within the length of time necessary for colonies to develop. If the ointment is changed in composition in such a way that it becomes a poorer solvent for the antiseptic substance, the partition coefficient is altered in a direction favorable to increased antiseptic effect.

Any other factor that would increase the rate of diffusion of the antiseptic substance from the ointment into the culture medium or tissues would likewise increase the observed antiseptic effect. For example, certain concentrations of cholesterol or similar substances in wool fat or other bases might increase the rate of diffusion.

*Various surface active agents* increase the antiseptic effect of ointments. Thus, Berthet (1937) found that Aerosol OT (dioctyl sodium sulfosuccinate) in 1:2000 concentration increased the bactericidal effect of merthiolate, mercurochrome, mercuric iodide, hexylresorcinol and other bactericides. Gregg and Zopf (1951) showed that the bactericidal potency of hexachlorophene is increased in presence of Tween 80 (polyethylene sorbitan mono-oleate). As previously indicated, the surface active agent may increase the rate of diffusion of the antiseptic substance from the ointment. Some workers have found that surface active agents increase skin penetration when incorporated in animal fats or emulsion bases but not with hydrocarbon bases. Other hypotheses which have been proposed to explain the increased effect of antiseptics in presence of surface active agents are: (a) chemical combination between the surface active agent and the antiseptic may form a more active product, (b) the surface active agent may facilitate penetration of the antiseptic into the bacterial cell membrane, (c) the surface active agent may promote the concentration of the antiseptic on the bacteria.

In using surface active agents in ointments it must be remembered that these substances may favor the development of molds. Thus it was observed by Bolle and Mirimanoff (1950) that ointments containing non-ionic detergent bases became moldy rather quickly. In such cases the use of an anti-molding agent is necessary.

*Effect of Water.*—Breslauer (1895) reported that the addition of water to a base increased its value as an antiseptic carrier. However, Husa and Radin (1932) found that the addition of 5 or 10 per cent of water had no significant effect on the width of the zone obtained in the agar-plate method with an ointment consisting of 5 per cent of phenol in a base containing 3 parts of petrolatum and 1 part of anhydrous wool fat. Since that time there have been many published reports indicating that in some cases the presence of water increased the antiseptic effect of ointments while in other cases water had no beneficial effect. An analytical evaluation of the results leads to the general conclusion that water present as an oil-in-water emulsion increases the antiseptic effect of water-soluble antiseptics. When the water is present as a water-in-oil emulsion or where the antiseptic is insoluble in water, the presence of water usually does not increase the antiseptic effect. This conclusion is readily understandable if one considers the situation which obtains when a layer of ointment is placed upon an agar gel in the agar-plate method. If the ointment has a grease base or water-in-oil base, there is a true interface between the ointment and the agar gel, since on the ointment side of the junction the grease or oil phase

is continuous, while on the agar gel side the aqueous phase is continuous. But in case of oil-in-water bases or mucilage bases the ointment and agar gel both have surfaces in which the aqueous phase is continuous and so there is not a true interface, *i.e.*, junction of two different phases of a system. Thus a water-soluble antiseptic present in the aqueous external phase of an ointment can readily diffuse into the aqueous agar gel. This situation is different than in case of a water-soluble antiseptic present in the internal phase of a water-in-oil emulsion base; in the latter case the aqueous phase of the ointment is separated from the aqueous phase of the agar gel by a barrier of oil or fat.

*Effect of Waxes.*—Cheyne (1915) observed that the addition of wax to the lanolin used as a base for 20 per cent phenol ointment reduced the antiseptic value by about one-fourth. Experiments by Husa and Radin (1932) showed that the use of 5 per cent of yellow wax in phenol ointments having a base of petrolatum and wool fat reduced the antiseptic properties to zero in the agar-plate test; these results were substantiated by Bryan (1936). It was also found that the addition of yellow wax to replace part of the base in 2 per cent ointments in benzoinated lard decreased the antiseptic efficiency (Craw and Lee, 1938). Subsequent research indicates that white wax, spermaceti and other waxes decrease the antiseptic effect of ointments containing various germicides, including chlorothymol (Clark, 1939; Burnside and Kuever, 1940). Thus it has been demonstrated conclusively that waxes generally reduce the antiseptic potency of ointments containing antiseptics.

*Effect of pH.*—Working with ammoniated mercury, sulfathiazole and calomel in hydrophilic (washable) bases, it has been found that a lowering of the pH increases the antiseptic effect (LeMar and White, 1944). These tests were made by the agar cup-plate method which is the same as the agar-plate method except that a circular depression or cup is produced in the center of the agar slab.

*Insoluble Inorganic Compounds.*—The antiseptic effect of ointments of insoluble substances such as zinc oxide and calomel does not appear to be greatly affected by the type of base or by the presence of water. Some of these compounds give very small zones with a variety of bases while others, *e.g.*, ammoniated mercury, consistently give large zones in all types of bases.

A significant factor for insoluble compounds was uncovered by Vicher *et al.* (1937). They showed that the fineness of subdivision of the insoluble compound was a deciding factor in the antiseptic effect. Using a specially prepared colloidal calomel, they obtained zones of 7 to 12 mm in the agar-plate method, compared with zones of 0.5 to 2 mm with the official grade of calomel. Later Maney and



Kuever (1944) found that finely divided mercurous iodide made by precipitation gave zones in the agar-plate method but no zones were obtained with ordinary official mercurous iodide. Hence the antiseptic effect of insoluble inorganic compounds appears to depend primarily on their toxicity to bacteria and on the fineness of subdivision, which exposes more surface of the particles and increases the solubility. For such colloidal or near-colloidal substances the use of a water-in-oil type of base has been found preferable to other bases.

*Sulfonamides* give larger zones of inhibition with oil-in-water emulsion bases than with hydrocarbon bases or water-in-oil bases (Bandelin and Kemp, 1946). Huyck *et al.* (1946) found that diffusion of sulfonamides was more rapid from oil-in-water bases than from water-in-oil bases; the addition of about 10 per cent of the surface active agent Aerosol OT increased the rate of diffusion from water-in-oil bases. Numerous other papers lead to the same general conclusion that oil-in-water bases are preferable for sulfonamides in the opinion of a majority of investigators.

*Antibiotics.*—The results of agar cup-plate tests and other methods by various investigators lead to the general conclusion that oil-in-water bases are preferable for penicillin ointments; it is necessary to keep the ointments under refrigeration to retard deterioration of penicillin in the presence of water. Bacitracin is unstable in ointment bases containing water or carbowaxes but is stable in a base consisting chiefly of polyethylene glycol diesters. Diffusibility of bacitracin and polymixin is much better from this base than from grease bases (Jeffries *et al.*, 1952).

The results cited illustrate the fact that in selection of ointment bases for antibiotics as much consideration must be given to the stability of the antibiotic in the base as to the ability of the base to release the antibiotic.

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## 17

# ANTISEPTIC POWDERS

## INTRODUCTION

ANTISEPTIC powders are impregnated with one or more antimicrobial substances and are intended for use directly or after solution in a liquid. Only those powders which are applied directly to living tissue for their bacteriostatic or fungistatic effect, or both, are considered here. Such formulations may contain one or more active ingredients in concentrations varying from 0.02 to 100 per cent. Various vehicles or diluents are employed according to the specific purpose intended for each.

Various antimicrobial agents have been employed for a variety of purposes, such as dusting powders, insufflations, surgeons' gloves, etc. and for the treatment of different kinds of infections such as epidermophytosis, skin rashes, etc. The value of most of these formulations has been determined primarily by clinical experience, their effectiveness for specific purposes being observed under conditions of use. In fact, clinical effectiveness has in most instances been the only criterion of the antimicrobial property of antiseptic powders widely employed in the past, at least during the period before laboratory test methods were available for the purpose.

## COMPOSITION OF ANTISEPTIC POWDERS

A survey of 114 antiseptic powder formulations which have been and are now being used for topical application shows an extensive list of ingredients, both diluents and active agents. The ingredients in those formulations which have been recommended for clinical use together with the range in per cent concentration employed are given in Table 58. The number of times those ingredients appear in different formulas is also listed. This list is presented



to illustrate the kinds of ingredients, both active and inert, used in various formulations of antiseptic powders.

Some antiseptic powder formulas, both proprietary and others which have been reported in the literature, contain ingredients which are not listed in the table below, and new formulations have recently been developed in response to special needs which contain

TABLE 58.—INGREDIENTS IN 114 ANTISEPTIC POWDER FORMULAS

<i>Ingredient</i>	<i>No. of times ingredient appears</i>	<i>Range in % con- centration</i>	<i>Ingredient</i>	<i>No. of times ingredient appears</i>	<i>Range in % con- centration</i>
Acacia	1	40	Paraformaldehyde	2	0 2-6
Alum	7	5 60	Penicillin	7	
Alum, Exsiccated	4	1 96	Perfume	3	qs
Aluminum Chloride	1	3	Petrolatum	1	2 3
Ammoniated Mercury	1	2	Phenol	3	0 38-1
Anthrasol	1	5	Proflavine Sulfate	1	1
Asbestos	1	50	Purified Infusorial Earth	2	10 59
Bentonite	9	9 71	Salicylic Acid	23	0 5 25
Benzoic Acid	4	1 2 5	Scarlet Red	2	10
Bismuth Subgallate	2	20	Silver Nitrate	1	0 6
Bismuth Subnitrate	7	25 70	Sodium Hexametaphosphate	1	5
Boric Acid	62	2 100	Sodium Perborate	2	15
Calamine	2	25	Sodium Thiosulfate	3	20
Calomel	6	25 50	Starch	25	10 88
Camphor	8	0 5-12	Sulfanilamide	2	67 100
Charcoal, Wood	1	50	Sulfathiazole	7	5-99
Chlorothymol	1	0 04	Talc	53	10-98
2:7 Diaminoacridine HCl	1	1	Tannic Acid	8	5-50
Ethyl p-aminobenzoate	3	5 20	Tannoform	1	33
Fuller's Earth	1	25	Thymol	7	0 1 1
Iodoform	3	25 50	Thymol Iodide	4	1 33-90
Kaolin	3	10 99 98	Tinct. Benzoin	2	8 10
Lead Stearate	1	9	Undecylenic Acid	2	2
Lycopodium	3	15 25	Wool Fat-Hydrous	1	2
Magnesium Carbonate	6	2 33 33	Zinc Carbonate	2	10 23
Menthol	9	0 5 2	Zinc Oleostearate	1	25
Methyl Rosaniline	1	10	Zinc Oxide	29	5 50
Oil Eucalyptus	1	0 2	Zinc Peroxide	6	10-20
Oil Rose Geranium	4	0 1 0 2	Zinc Stearate	15	1 5-80
Oil Thyme	1	0 2	Zinc Undecylenate	2	20
Oil Theobroma	1	6			
Orris Root	2	10 33			

ingredients not given here. The ingredients listed, however, may be useful as background material in understanding the general nature, formulation possibilities, and basic features of antiseptic powders.

Results of *in vitro* tests have not been reported on formulations composed of most of these active ingredients. Studies on bacteriostatic and fungistatic activity by suitable laboratory tests are helpful in determining or at least estimating the antiseptic possibilities of various compounds. Now that suitable *in vitro* tests are available, such determinations would present useful information in this connection. Brief descriptions of such tests are given here for con-

venience, although they are discussed in greater detail in the chapters on the testing of antiseptics and fungistats.

## TEST METHODS

*Bacteriostatic Test Method.*—The test for antiseptic powders developed by Reddish (1927) has been found satisfactory for this purpose and is conducted as follows:

Liquefy 100 ml of nutrient agar and cool to 45° C; add to the melted agar 1 drop of 24-hour broth culture of *Micrococcus pyogenes* var. *aureus*, of normal phenol resistance, and mix well. Transfer 20 ml portions into 9 cm sterile petri dishes and allow to solidify. With sterile spatula, apply approximately 1 g of the test powder to the surface of the agar in the center of the plate and cover with a sterile unglazed clay top. Incubate the plates, top up, at 37° C for 48 hours. Measure the zone of penetration and inhibition from the edge of the powder to the edge of the clear zone. The clear zone of inhibition indicates the degree of bacteriostatic potency of the antiseptic powder.

To determine the effect of organic matter on bacteriostatic activity, add 20 ml of sterile horse serum (without preservative) to the 100 ml of nutrient agar cooled to 45° C, and repeat the test as described.

A slight modification of this test is specified for determining the antiseptic potency of Compound Zinc Sulfate Powder, N.F. by the National Formulary Committee and is described in the National Formulary (1950) 9th ed, p 575.

*Fungistatic Test Method.*—The method described by Burlingame and Reddish (1939) as specifically applied to antiseptic powders is conducted as follows:

Liquefy Sabouraud's agar containing maltose and cool to 45° C. Transfer 20 ml portions into 9 cm petri dishes and allow to solidify. By means of a sterile cotton swab<sup>1</sup>, spread evenly on the surface of the agar a culture of *Epidermophyton interdigitale*<sup>2</sup> grown on slants of Sabouraud's agar for 5 days at room temperature. With sterile spatula, apply approximately 1 g of the test powder to the

<sup>1</sup>To obtain proper distribution, the swab should be a long loose one, the cotton extending an inch beyond the end of the applicator stick.

<sup>2</sup>Investigations as to fungistatic activity should be made on more than one test organism. Burlingame and Reddish used *Trichophyton interdigitale*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton rosaceum*, and *Epidermophyton floccosum*. Fungi actually involved in fungous infections are used.

*Note.*—Methods for determining sterility of crystalline or powdered solids are specified in the United States Pharmacopeia (1950).

surface of the agar in the center of the plate and cover with a sterile unglazed clay top. Incubate the plates, top up, at room temperature for 1 week. Measure the zone of penetration and inhibition from the edge of the powder to the edge of the clear zone. The clear zone of inhibition indicates the degree of fungistatic potency of the powder.

To determine the effect of organic matter on the fungistatic activity, add 20 ml of sterile horse serum (without preservative) to 100 ml of the Sabouraud's agar containing maltose and cooled to 45° C. and repeat the test as described.

The methods just described are useful for several purposes, such as for screening experimental formulas, determining penetration and inhibitory properties, estimating the effect of organic matter, etc. Although results from these tests cannot be interpreted directly in terms of clinical values, they are useful for estimating probable value under conditions of use.

Meyer (1940) suggested a test for determining the fungistatic action of powders. In this test, a slurry of the powder is made in water before adding to a narrow slot in the inoculated agar plate. The powders under discussion here are those used in the powder state under the conditions of practical use, and they do not ordinarily have sufficient moisture to produce the equivalent of a slurry. The Meyer test method is not considered suitable for the purpose of testing antiseptic powders which are recommended for direct applications.

Weidman, Emmons, Hopkins, and Lewis (1945) have recommended an *in vitro* fungicidal test. Water insoluble substances are put into solution in acetone or propylene glycol and used in a modified phenol coefficient test. The test organism is a pathogenic fungus etiologically related to the fungus diseases of the skin. This method does not simulate in any degree the actual conditions of use.

Cade (1947), using a filter paper disc method, has attempted to prove that the size of the zone of inhibition is not necessarily the criterion by which the effectiveness of the material may be judged. These studies were made with filter paper discs wetted with the solution under test and placed on inoculated agar plates. The variables in the zone test technique are listed as follows:

- (a) The type of procedure—cup or disc.
- (b) The diameter of the cup or disc used.
- (c) The composition and depth of the agar and its effect upon the diffusability of both chemical and organism through the agar.



- (d) The absorptive or retentive properties of the disc.
- (e) The amount of material on the disc or in the cup.
- (f) The manner of incubation (time and temperature)
- (g) The vehicle used to carry the chemical.
- (h) The type of test organism, its age, pathogenicity, inoculum size, etc.
- (i) The pH of the agar of the test solution.

In spite of these variables, the agar plate tests as described above are useful for the purposes indicated. Fredell, Burlingame, and Reddish (1952) made use of these tests in a study of the bacteriostatic and fungistatic activity of certain experimental antiseptic powder formulations. The results of these tests are given in Table 9.

The fungistatic results obtained with one series of formulations are illustrated in Figures 10, 11, 12, and 13, in which 4, 8, 16, and 5 per cent salicylic acid in talc were used. The effects of increased concentrations of this active ingredient are easily observed and measured. This demonstrates the value of the test method in measuring fungistatic activity under *in vitro* conditions.

## COMMENTS

It is evident from the results given in Table 59 that antiseptic activity of certain active ingredients in powder vehicles depends largely on the concentration employed. Also certain ingredients are more active against pathogenic fungi other than bacteria than against certain bacteria. In some instances the bacteriostatic and fungistatic action is low due to the character of the diluent. This is often due to the extremely low solubility of these agents. In any case, these results illustrate the value of the test method in determining antiseptic activity of the formulations used in these tests.

As indicated previously, such *in vitro* studies have not been made on most of the formulations investigated and summarized in Table 59. The results of a study of a few special formulas shown in Table 59 show quite clearly the need for such *in vitro* determinations.

## GENERAL CONSIDERATIONS

Because of the very serious lack of published data on antiseptic powders, it is practically impossible to evaluate the bacteriostatic and fungistatic activity of the formulations commonly used in practice. Such laboratory tests have no doubt been conducted on certain proprietary antiseptic powders, but in most instances the results



Fig. 10.—Salicylic acid (4 per cent) in talc on Sabouraud's agar showing penetration and fungistatic activity against *T. interdigitale*.

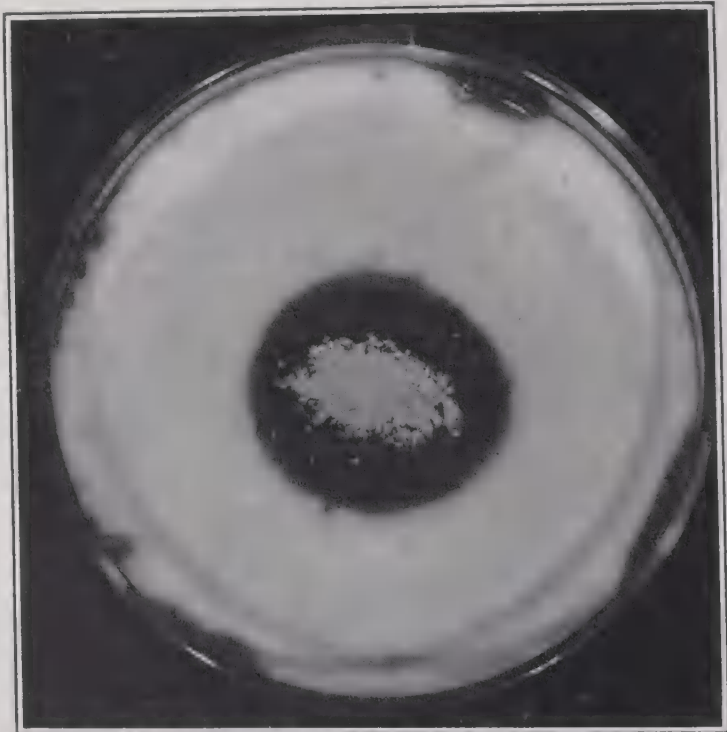


Fig. 11.—Salicylic acid (8 per cent) in talc on Sabouraud's agar showing penetration and fungistatic activity against *T. interdigitale*.

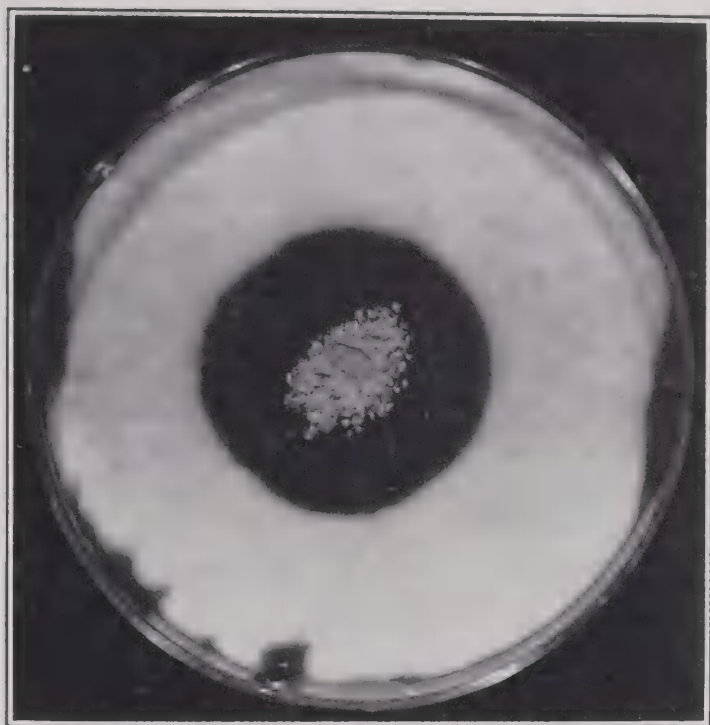


Fig. 12.—Salicylic acid (16 per cent) in talc on Sabouraud's agar showing penetration and fungistatic activity against *T. interdigitale*.

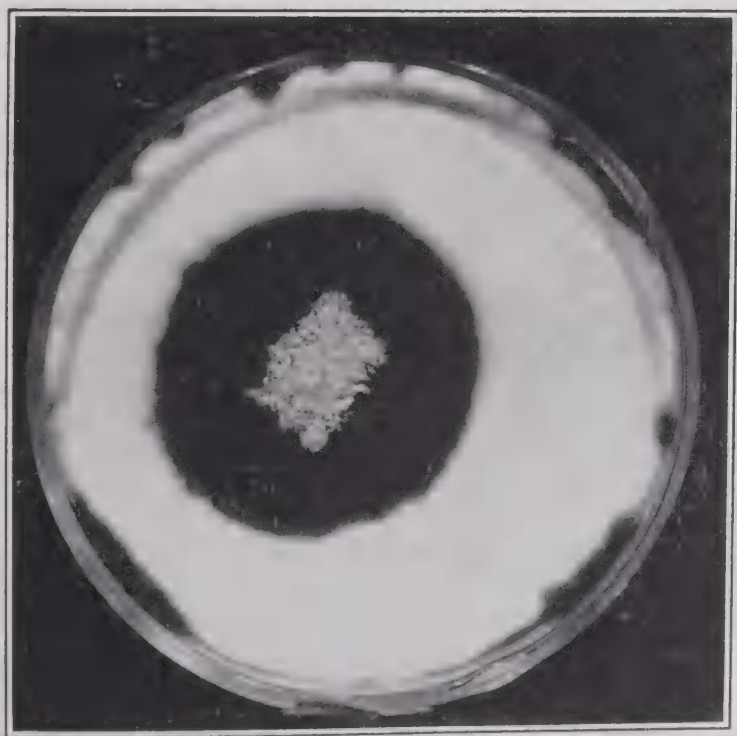


Fig. 13.—Salicylic acid (25 per cent) in talc on Sabouraud's agar showing penetration and fungistatic activity against *T. interdigitale*.



have not been published. For this reason the basic information presented may suffice for background information.

There is need for more fundamental data on the bacteriostatic and fungistatic properties of compounds used in antiseptic powders. Also studies should be conducted relative to the effect of various diluents or vehicles on the active ingredients. It is evident that

TABLE 59. — BACTERIOSTATIC AND FUNGISTATIC ACTIVITY OF SOME ANTISEPTIC POWDER INGREDIENTS

Powder	Zone of penetration and inhibition <sup>1</sup> in cm		
	A <sup>2</sup>	B <sup>3</sup>	C <sup>4</sup>
Benzoic Acid 1.0% in tale	0.0	0.4 part	—
Benzoic Acid 2.5% in tale	0.0	1.5	0.8
Boric Acid 10% in tale	0.5	3.0	2.5
Boric Acid 25% in tale	0.5 Cl, 0.9 part	3.5 NoG	3.5 NoG
Boric Acid 50% in tale	0.7 Cl, 1.0 part	3.5 NoG	3.5 NoG
Boric Acid 75% in tale	1.0 Cl, 1.2 part	3.5 NoG	3.5 NoG
Boric Acid 100%	1.0 Cl, 1.2 part	3.5 NoG	3.5 NoG
Menthol 2% in tale	0.0	—	—
Phenol 1% in tale	0.0	0.0	—
Salicylic Acid 4% in tale	0.2	0.3	0.3
Salicylic Acid 8% in tale	0.5	0.9	1.3
Salicylic Acid 16% in tale	0.7	1.0–1.4	1.5
Salicylic Acid 25% in tale	0.6	2.0	1.7
Sodium Thiosulfate 20% in tale	0.0	0.8–1.0	0.7
Tale 100%	0.0	0.0	—
Thymol 1% in tale	0.4 Cl, 1.7 part	3.5 NoG	2.8
Thymol Iodide 25% in tale	0.0	0.0	—
Thymol Iodide 50% in tale	0.0	0.0	—
Thymol Iodide 100%	0.0	0.0	—
Zinc Oxide 12.5% in tale	0.0	0.0	—
Zinc Oxide 25% in tale	0.0	0.0	—
Zinc Oxide 50% in tale	0.0	0.0	—

<sup>1</sup> Cl—clear; part—partial; NoG—no growth on plate

<sup>2</sup> Bacteriostatic zone against *M. pyogenes* var. *aureus* in nutrient agar.

<sup>3</sup> Fungistatic zone against *T. inguinale* on Sabouraud's maltose agar.

<sup>4</sup> Fungistatic zone against *T. interdigitale* on Sabouraud's serum maltose agar.

much information is needed in order to properly evaluate and classify the formulations recommended for the purpose. This is especially so because of the wide use of antiseptic powders. In addition, there is need for further studies on the correlation of results obtained by *in vitro* tests with clinical effectiveness. For the present, at least, the basic material presented here may be useful in a general understanding of the principles involved and the possibilities of future studies.

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## 18

# VIRUCIDAL AGENTS

## GENERAL CONSIDERATIONS

VIRUSES are susceptible to inactivation by many agents, physical, chemical and biological. The latter include the antibodies and other components of the defense mechanisms by which the host combats infection. These invaluable virucidal agents are outside the scope of this chapter. Virus inactivation by all other agents takes place to a significant extent only outside the cells in which the virus multiplies, as shown by the fact that no therapeutic agent has been found that penetrates these cells and cures a virus infection. It should be mentioned, however, that this lack of curative agents does not apply to diseases caused by the Rickettsiae, which do not come within the virus category as generally accepted.

Many of the studies dealing with the action of virucides have not been designed to elucidate their mode or rate of action, but rather have dealt with the destruction of infectivity under a single set of experimental conditions of time, temperature, pH, concentrations of virus and agent, etc., or with only a few variations of one or two of these. Even the degree of infectivity of the original virus suspension frequently had not been determined. Some of these studies were made with the primary object of determining the immunological properties of inactivated virus and so the destruction of infectivity was incidental to the main purpose of the experiments.

Complete inactivation of virus suspensions cannot be determined because of a serious limitation inherent in the methods used to detect active virus. These depend upon observing whether the suspensions cause recognizable infections in the most susceptible hosts available for experimental use. The number of active virus particles required to initiate infection varies with the host and the virus and even with the strain and previous history of each.



This number of elementary bodies remains unknown, though approximations have been obtained in some instances. So, the rate of decrease in activity and the conditions under which a reasonable number of samples no longer causes infection can be determined with accuracy; but the elimination of the last active virus particle cannot be detected. The term complete inactivation has been used frequently to indicate a degree of inactivation only to a point below the level of infectivity.

The rate of inactivation of viruses has been studied under different conditions. In most instances, but not all (Lauffer and Geller, 1950), the reaction is of the first order with respect to virus. Thus, there is a direct relation between the time during which the virus is exposed to the inactivator and the logarithm of the concentration of active virus remaining. It follows from this that the amount surviving is in direct proportion to the initial virus concentration. If 10 per cent of the activity remains after treatment with an agent for 15 minutes, 1 per cent remains after 30 minutes, and 0.1 per cent survives at 45 minutes. Taking this rate of inactivation, if in each volume that is injected into a test animal the number of 50 per cent infective units of virus is originally 100, some of the animals that receive virus exposed for 30 minutes will be infected, but none after a 45-minute reaction period. If this experiment had been conducted, as has been the case of numerous tests reported in the literature, with a single exposure period that destroyed infectivity, 45 minutes in the above example, the results would give a qualitative indication that the agent is capable of inactivating the virus; but they would lack the quantitative relationships necessary for application to a suspension with another degree of infectivity. In fact, in this experiment if the initial suspension had contained 10 times as many 50 per cent infective units of virus, about half of the test animals that received virus exposed for 45 minutes would have been infected.

Many of the experiments designed to determine the action of virucidal agents have been hampered by the presence of large amounts of other organic material present in the mixtures. These agents, lacking the specificity of antibodies, readily combine with some of the extraneous substances and so are diminished in their effective concentration or are blocked in other ways, that will be mentioned later, from exerting their action on the virus. As viruses multiply only within the cells of a susceptible host, the initial suspensions prepared by triturating infected tissue contain small amounts of virus compared to the large quantity of material derived from the host cells. Relatively pure suspensions of virus can be derived from such mixtures only by elaborate and precisely regu-

lated procedures including differential centrifugation with forces up to about 60,000 times gravity. Purification of the few viruses that multiply in the allantoic membrane of chick embryos is more feasible. Viruses in this group, which includes those causing influenza and mumps, proliferate in the cells lining the allantoic cavity and then are extruded into the allantoic fluid. This fluid consists of the excretions of the embryonic kidneys. At 13 days of incubation, approximately 75 per cent of the nitrogen in allantoic fluid is due to the presence of uric acid, the chief renal excretory product of birds. Yet the concentration of uric acid at this age is only about 25 mg per ml (Romanoff, 1952).

Mammalian viruses differ widely in their chemical composition. Proteins account for about 90 per cent of the dry weight of rabbit papilloma virus, but less than 50 per cent of influenza A virus. About 10 per cent of swine influenza virus consists of carbohydrate, while vaccinia virus contains less than 3 per cent. Nearly 50 per cent of influenza A virus is composed of lipid compounds, though less than 6 per cent of the dry weight of vaccinia virus is due to this group of compounds. Most of the other viruses come within these broad ranges. These differences in the proportions of large groups of compounds that make up the virus particles would lead one to anticipate the divergent rates of destruction of their biological properties exhibited by different viruses when exposed to the influence of certain physical and chemical conditions.

### SPONTANEOUS INACTIVATION

In general, viruses do not survive in nature for prolonged periods after the host has died or they have been separated from susceptible living cells. The speed with which they lose activity depends on which virus is involved and the conditions surrounding the virus, such as the hydrogen ion concentration, the enzymes present, temperature, etc. Influenza A virus in low concentration in normal chick embryo allantoic fluid becomes noninfective when stored for 24 hours at 35°C (Rasmussen and Stokes, 1951). Similarly, mumps virus diluted in allantoic fluid so that each volume to be tested in eggs contains 100 LD<sub>50</sub> loses its infectivity in 6 hours at 25°C (Eaton, Cheever and Levenson, 1951). The addition of normal serum usually decreases the rate of this type of inactivation.

Yellow fever virus is capable of surviving in the body at infectious levels for several hours after death. This was first shown by Bauer (1931) who took samples of blood at intervals from an infected monkey that was kept after death at a temperature close to 28°C. After 9 hours, 0.01 ml of blood, but not 0.0001 ml, was found to be infectious for other monkeys. At this time the organs

were described as being in an advanced state of decomposition. It should not be assumed, however, that tissues of animals dying of other virus diseases remain infectious for as long a time under comparable conditions. Blood from monkeys infected with yellow fever virus is more highly infectious than that of animals infected with most other viruses. This may be because the blood contains an unusually high concentration of virus or because only a small number of virus particles is required to initiate infection or, what is more likely, because of a combination of these two factors. In other experiments Bauer found that the  $LD_{50}$  of blood from monkeys infected with yellow fever virus was 1.0 ml of a dilution of  $10^{-9}$ . In view of this, there had probably been about a millionfold decrease in active virus during the 9 hours after death.

Some conditions which simulate those that might occasionally be present in nature permit the survival of active virus for longer periods. Under certain circumstances, viruses dried at room temperature retain their activity for many days. The period of survival depends to a large extent on the presence of other substances in the suspension. The effect of drying influenza A virus under various conditions comparable to those that may prevail near a patient with the disease was studied by Parker, Dunham and MacNeal (1944). They sprayed infected allantoic fluid on sterile talc and glass, and caused it to dry in periods extending to 2 hours. Active virus could be recovered 1 to 4 and rarely 15 days later. The addition of 0.25 per cent mucin had a marked protective action, permitting the virus to survive for periods up to 45 days. The fact that influenza virus can persist for many days in mucoid material has obvious implications as to its survival on contaminated drinking and eating utensils. Edward (1941) has shown that influenza virus can be recovered from a dry cloth 2 weeks after it has been saturated with a suspension of infected mouse lung. A practical and probably the first application of this type of procedure was made by the Chinese many centuries ago. They found that material taken from the lesions of smallpox retained its activity for variolation when dried on splinters of bamboo. This virus, however, and the almost identical virus of vaccinia are unusually resistant to inactivation. A suspension of vaccinia virus in buffered saline solution does not undergo more than a 1000-fold decrease in titer on standing for months at  $37^{\circ}\text{C}$  (Buddingh and Randall, 1951).

## RETARDED INACTIVATION

The subject of inactivation of viruses is intimately linked with the conditions under which spontaneous inactivation can be retarded. A few of these will be mentioned briefly.



*Desiccation.*—Mention has been made of desiccation at room temperature as a procedure that is sometimes effective in delaying inactivation. Even so, there is considerable loss of virus during the drying process. Desiccation of frozen suspensions causes far less inactivation and permits more complete drying. The material is placed in an ampule and frozen in the form of a layer covering the inner wall of the container. This is accomplished by rotating the ampule in a nearly horizontal position while it is immersed in a mixture of alcohol and solid carbon dioxide. The sole function of the alcohol is to make good contact with the glass and so to transmit heat rapidly from the suspension. The ampule is then attached to a vacuum line with a desiccant chamber between it and the pump. If the pressure is 200  $\mu$  or less, the drying proceeds so fast that heat from the surrounding air does not cause melting of the suspension. This heat is removed as the latent heat of vaporization. When drying is complete, the ampule can be sealed without breaking the vacuum. Under these conditions viruses retain their activity for many months or years with little loss of activity.

*Glycerol.*—A simple method for decreasing the inactivation rate of viruses is storage in 50 per cent glycerine at refrigerator temperature. This is particularly convenient for the preservation of virus in small pieces of infected tissue. The effectiveness of glycerol probably depends on its hygroscopic property and so its action is comparable to a partial drying of the virus. Influenza virus in ferret turbinates kept in 50 per cent glycerol retains its activity for at least a year. Mouse encephalomyelitis virus has remained active for 4 years in the author's laboratory when infected mouse brains were stored in 50 per cent glycerol at about 4°C. The most extensive use of this procedure is in the preservation of vaccinia virus for immunization.

*Low Temperatures.*—The rate of spontaneous inactivation of viruses is decreased by lowering the temperature. At refrigerator temperature, 0° to 4°C, measles virus retains its infectivity for monkeys for several days (Rake and Shaffer, 1940) and influenza virus in allantoic fluid remains infective for eggs for at least 6 weeks.

Though the freezing process in itself destroys some virus, frozen preparations retain activity for prolonged periods. In addition to the lowering of the rate of natural inactivation caused by the decreased temperature, freezing separates the virus from water. When a solution freezes, practically pure crystals of ice are formed leaving an ever increasing concentration of the solute in the interstices. The freezing of a virus suspension may thus be considered in part as a form of drying, the extent of removal of water depend-

ing on the temperature. Most viruses can be kept for many months at  $-20^{\circ}\text{C}$  in a mechanical freezer or for years at about  $-70^{\circ}\text{C}$  in a chest containing solid carbon dioxide.

*Serum.*—Various biological preparations retard the natural deterioration of viruses. Among these the most frequently used is normal serum. An example is the extensive use formerly made of serum in yellow fever vaccine to maintain the activity of this attenuated virus. A quantitative study with the Ilhéos encephalitis virus has shown the striking difference in the rate of inactivation of a suspension of the virus when one portion was diluted in physiological saline solution alone and another portion in saline solution containing 10 per cent human serum. After standing at room temperature for 5 days, the loss of virus activity in the salt solution was 100,000 times greater than in the diluent containing serum (Koprowski and Hughes, 1946).

## ACCELERATED INACTIVATION

### PHYSICAL AGENTS

*Elevated Temperatures.*—The rate of inactivation of viruses steadily increases with a rise in temperature. There is no break between the rate of spontaneous inactivation at room temperature and the rapid rate that occurs at  $60^{\circ}\text{C}$ . Because of this relationship, the process here termed for simplicity spontaneous inactivation could be defined more precisely thermal inactivation. Lauffer, Carnelly and MacDonald (1948) showed that the inactivation proceeds at logarithmic rates that are characteristic for each temperature. Working with purified influenza A virus at pH 7, they found that a 100-fold decrease in active virus takes place in 60 minutes at  $5^{\circ}\text{C}$  and in 2.5 minutes at  $56^{\circ}\text{C}$ .

An early study on the inactivation of a virus, vaccinia, was made by Henry (1831). From these experiments he concluded that the infectious matter of cow-pock" is not "rendered inert by a temperature below  $145^{\circ}\text{F}$ " ( $62.8^{\circ}\text{C}$ ). It seems likely that uniform heating of the vessel and contents did not take place, as Gordon (1925) obtained destruction of infectivity at  $55^{\circ}\text{C}$  in 30 minutes when calf lymph containing 10,000 MLD was sealed in capillary tubes and submerged in a water bath. Some active virus remained after heating for 30 minutes at  $50^{\circ}\text{C}$ . In general, suspensions of viruses have been found to lose their infectivity on being heated for 30 minutes at  $56^{\circ}\text{C}$  (van Rooyen and Rhodes, 1948). The presence of normal serum slows the process.

The figures cited in this section represent inactivation under laboratory conditions. More prolonged heating or higher tem-

peratures would have to be used if the desired temperature could not be attained promptly throughout the infective material.

*Filters.*—Diatomaceous earth and silicic acid filters in general do not free material of viruses even though they may be highly effective in preventing the passage of bacteria. A reduction in the amount of virus present in the filtrate, however, is brought about by two important factors. One is the removal of coarse particles of matter containing virus and the other is the adsorption of virus elementary bodies themselves on the walls of the pores of the filter. The latter applies particularly to the larger viruses, such as that of vaccinia which is retained to a considerable extent by Berkefeld V filters even though both it and the filter carry negative electrical charges. Certain conditions diminish the amount of virus that is adsorbed by a filter. One of these is the passage of broth through the filter before the introduction of the virus suspension. Also, prolonged use without cleaning coats the walls of the pores of the filter with virus and other substances so that a greater proportion of the virus passes through.

*Ultraviolet Radiation.*—Viruses are readily inactivated when exposed to ultraviolet light of suitable wave length and intensity. The greatest sensitivity of influenza virus occurs at about 2650 Å, with little diminution at 2537 Å (Hollaender and Oliphant, 1944). The quantity and composition of the intervening substances impose limitations on the effective range of radiation. In air this range is far greater than in liquids, though the presence of dust may protect some virus particles from adequate exposure. Ultraviolet light has been used extensively to inactivate viruses in the air, especially in crowded places such as schools and in hospitals where contagion is a serious problem. The methods used must avoid prolonged exposure of the eyes. This is accomplished by irradiating the recirculated air in the ducts of air-conditioning units or by irradiating the upper portion of the room and relying on convection currents to transmit the virus into the path of the rays. In order to isolate certain portions of a building, ultraviolet curtains can be installed across the doorways and halls. Such procedures have a definite value in decreasing the spread of virus infections.

Solutions of salts and organic compounds absorb ultraviolet light much more than distilled water. This decreases the depth of effective penetration by the rays. Transmission through 11 cm causes a 9 per cent loss of intensity in distilled water, 32 per cent loss in physiological saline solution and 90 per cent loss in Ringer's solution. The distance that causes 50 per cent decrease of intensity is 0.074 mm in a rabies vaccine, 0.045 mm in 5 per cent albumen and 0.0063 mm in serum (Buttolph, 1953).



A simple procedure for inactivating viruses consists of placing 15 ml of the suspension in a petri dish on a rotator about 12 inches below a bactericidal lamp. If the suspension is infected allantoic fluid that has been dialyzed against buffered saline solution to remove the uric acid, destruction of infectivity is achieved in 3 or 4 minutes; but if dialysis has not been performed, the process may require as much as an hour. Such a procedure lacks uniformity as it depends on the circulation of the virus particles to near the surface for sufficient periods to permit inactivation. A more reliable procedure is to allow the suspension to pass as a film through the path of the rays. This can be accomplished by having the suspension run slowly down the inside of a quartz tube set diagonally or by feeding it to the bottom of an inverted conical chamber that is rotating fast enough to make the suspension creep up the inner surface. The thickness of the film and the period of exposure can be regulated by changing the rate of feed and the force that makes it move. Besides the uniformity of exposure, an advantage is that this is a continuous process and can be used on a large scale.

*Vibration.*—High frequency vibrations, 9,000 to 800,000 cycles per second, have little effect on crude suspensions of virus or on purified virus to which serum has been added. When a minimum of extraneous material is present, however, viruses are subject to inactivation by this means. Hamre (1949) obtained more than a 1,000-fold decrease in active virus when she exposed a highly purified suspension of influenza A virus for 1 hour to 800,000 cycles per second with 765 watts.

#### INORGANIC AGENTS

*Chlorine.*—The virucidal property of chlorine was discovered early (Central Board of Health, 1831). In an experiment for which Faraday provided the chlorine, "smallpox matter, both on glass and on linen cloth" was exposed for 3 hours to atmospheres containing chlorine gas in the proportions of 1:25 and 1:50. The material was then rubbed into the scarified skin of 7 individuals including 2 who "were inoculated in the usual manner in six places" for variolation. "All of these failed to produce any disease. . . . All these seven persons have since been vaccinated, and have had the disease in the most perfect and regular way."

The viruses of fowl pox and laryngotracheitis were found by McCulloch (1945) to be readily inactivated in F. D. A. broth containing available chlorine to the extent of 50 ppm. The virucidal action of chlorine solutions is greatly reduced by the presence of many biological compounds, such as proteins, that are subject to oxidation or combination with chlorine. Under certain conditions,

however, even if measures are not taken to meet the chlorine demand of such compounds by adding chlorine to give a fixed level of available chlorine, these substances may actually increase the virucidal action of the chlorine. This applies if the substances by their buffer action or acidity reduce the pH of an otherwise alkaline solution to within the range of greater chlorine effectiveness (McCulloch, 1945).

One of the most valuable uses of chlorine would be in the destruction of poliomyelitis virus in swimming pools. Rhodes and van Rooyen (1953) conclude that it is probable that infection can be acquired by swimming in pools even though the water is chlorinated. The period elapsing between contamination of the water and exposure of a swimmer obviously cannot be controlled. Crowding would decrease this period. When partially purified poliomyelitis virus is suspended in water from lakes, rivers and wells and chlorine is added to yield at the end of the test period 0.05 ppm residual free chlorine, infectivity is destroyed in 10 minutes, provided the pH is within the range 7.9 to 8.3 (Lensen, Rhian, Stebbins, Backus and Peterson, 1949). In lime-treated water, pH 10.0 to 11.25, from 0.1 to 0.15 ppm residual free chlorine are required to achieve the same results. A factor which limits the amount of chlorine that can be used in swimming pools is its irritant effect on the eyes of the swimmers. This action is closely related to the pH of the water. Results obtained by Mood, Clarke and Gelperin (1951) in a prolonged study using a pool under controlled conditions show that total available residual chlorine in a concentration of 0.5 ppm at pH 8.0 is less irritating to the eyes than is 0.05 ppm at pH 7.0. However, as was mentioned before, the virucidal power of chlorine decreases as the pH is increased.

*Copper Salts.*—The cupric ion has only a weak action on viruses. A 1 per cent, but not a 0.1 per cent, solution of copper sulfate destroys the infectivity of influenza A virus in allantoic fluid in 3 minutes (Dunham and MacNeal, 1944). Vaccinia virus, in conformity to its greater resistance to virucides, retains its infectivity under similar conditions. Yaoi and Kasai (1931) found that purified vaccinia virus in 1 per cent copper chloride retained infectivity for at least 30 minutes.

*Hydrogen Ion.*—Mammalian viruses, as might be anticipated from the environment in which they multiply, are in general most stable between pH 6 and 8. The rate of inactivation usually does not increase rapidly, however, as the pH deviates from this range. Moulder and Weiss (1951) showed that under certain conditions of storage the titer of feline pneumonitis virus remains essentially constant at pH 6.5 to 7.5 and in the same period decreases by 1 log

unit at pH 5 and at pH 9. Stock and Francis (1940) found influenza virus to be rapidly inactivated below pH 5 and above pH 10. Employing suspensions buffered to give several different pH values, Lauffer and Wheatley (1951) have shown that in the pH range 5.9 to 9.0 the rate of denaturation increases as the pH of the medium is lowered.

*Hydrogen Peroxide.*—Viruses are inactivated by hydrogen peroxide, but the presence of organic material that competes with the virus greatly decreases this action. Washed vaccinia elementary bodies are inactivated at room temperature by 0.1 per cent hydrogen peroxide at a rate that decreases the titer by about 4 log units in 90 minutes, but in the presence of 8 per cent normal rabbit serum the decrease is only about 2 log units (Rivers, Smadel and Chambers, 1937).

*Hydrosulfite.*—Sodium hydrosulfite, a strong reducing agent, inactivates viruses to a significant degree only when in relatively high concentration. Thus Yaoi and Kasai (1931) obtained destruction of the infectivity of a weak suspension of washed vaccinia virus in 30 minutes at 20° C in the presence of 2.5 per cent sodium hydrosulfite.

*Iodine.*—Weak solutions of iodine, 0.00055 N, have a rapid inactivating action on influenza virus; but even a small amount of organic material, such as that contained in chick embryo allantoic fluid diluted 1:10, decreases this action considerably (Dunham and MacNeal, 1944). Allantoic fluid from 11 day chick embryos contains about 15 mg uric acid per ml. This represents approximately three quarters of the total nitrogen in the fluid, urea accounting for an additional 8 per cent (Romanoff, 1952). The strength of iodine mentioned above is obtained by diluting a 7 per cent tincture 1:1000. Such a solution contains iodine in a concentration of 70 ppm. Iodine vapor in a concentration of 0.1 ppm inactivates at a recognizable rate influenza virus suspended in the air (Stone and Burnet, 1945).

*Mercury.*—Bichloride of mercury in a concentration of 1:1000 rapidly inactivates the influenza and vaccinia viruses. In 3 minutes such a solution destroys the infectivity of a suspension of vaccinia virus (Dunham and MacNeal, 1943). A 1:5000 solution inactivates a suspension of this virus in 1 hour (Gordon, 1925). In similar experiments with the agent diluted 1:10,000 and the vaccinia suspension diluted so as to contain 200 MLD, infectivity is destroyed also in 1 hour. Undoubtedly the dilution of other substances present increases the amount of mercuric ion available for action on the virus.

Sodium ethyl mercuri thiosalicylate, merthiolate, in a concentration of 1:10,000 has little effect on viruses. Suspensions of neuro-



tropic viruses containing this amount retain their infectivity for months (Casals, 1947).

*Potassium Cyanide*.—Potassium cyanide is a compound that is highly toxic for animals and for bacteria, but apparently has little action on viruses *in vitro*. Yaoi and Kasai (1931) exposed washed vaccinia elementary bodies to a 1 per cent neutralized solution of potassium cyanide for 48 hours at 15° C. Infectivity was retained. Titrations before and after the test period might have revealed partial inactivation. However, the contrast with other agents is emphasized by the fact that these authors found that under similar conditions 0.05 per cent mercuric chloride destroyed infectivity in 30 minutes.

*Potassium Permanganate*.—The virucidal action of potassium permanganate has been tested with several viruses. Most of these studies have been conducted without titrations of the virus that remained after various periods of exposure and so do not indicate the rate of action. Instead, the experiments were designed to show the concentration of the compound that is required to destroy infectivity under certain conditions. The presence of a considerable amount of non-virus organic material in the mixtures inhibits the action. In most instances, a 1:10,000 solution of potassium permanganate has been found to destroy the infectivity of virus suspensions in half an hour at room temperature.

*Silver Nitrate*.—The effect of silver nitrate on a highly purified suspension of influenza virus was tested by Knight and Stanley (1944). The suspension contained only about  $10^{-11}$  gm of nitrogen per ml as determined by the Nessler method. When this was mixed with silver nitrate solution in a final strength of 0.05 N, 0.85 per cent, such a rapid destruction of infectivity took place that an immediate titration revealed at least a millionfold reduction in titer.

*Sodium Chloride*.—Vaccinia virus, partially purified by adsorption on kaolin and elution, was tested by Yaoi and Kasai (1931) for its survival in graded concentrations of sodium chloride. The mixtures were kept for 7 days at 37° C before testing. Infectivity was destroyed by 20 per cent saline solution and partially so by both 10 per cent and 0.1 per cent saline solution. The greatest activity was retained in the presence of 0.85 per cent sodium chloride.

## ORGANIC AGENTS

*Acetone*.—Acetone inactivates viruses to a significant extent only in high concentration. A 25 per cent solution usually is required to destroy infectivity in half an hour.

*Acridine*.—Significant inactivation of viruses takes place in hours at 37° C in the presence of certain nitroacridine and chloroacridine compounds (Eaton, Cheever and Levenson, 1951).

*Antibiotics*.—Penicillin and streptomycin do not affect viruses. Advantage has been taken of this fact to separate viruses from bacteria in biological specimens. They have been used extensively at final concentrations of 500 units of penicillin and 1000  $\mu$ g of streptomycin to suppress bacteria in throat washings and similar contaminated material before injection into eggs for the recovery of viruses (McKee and Hale, 1947). Streptomycin alone in the high concentration of about 27,000  $\mu$ g per ml has been used successfully for this purpose even when only a small amount of virus was present in the throat washings (Lowell and Buckingham, 1946). Though this method does not prevent the growth of all bacteria in eggs, it does emphasize the innocuousness of streptomycin for influenza virus.

Cabasso, Moore and Cox (1952) found that crystalline aureomycin, 8 mg per ml, has a slight effect on vaccinia virus *in vitro*; but heat inactivated aureomycin has an even greater effect, indicating that this is not due to the factor that is responsible for the antibacterial action. A similar virucidal action of inactivated penicillin against three viruses has been reported by Groupé and Rake (1947).

Ehrlichin, an antibiotic produced by *Streptomyces lavendulae*, prevents death or the formation of hemagglutinin in chick embryos infected with a mixture of influenza B virus and low dilutions of the culture filtrates (Groupé, Frankel, Lechevalier and Waksman, 1951).

*Ascorbic Acid*.—Poliomyelitis virus was first shown by Jungeblut (1935) to be inactivated *in vitro* by ascorbic acid. Solutions of the vitamin containing 5 mg per ml and brought to pH 6.6 or higher destroy the infectivity of suspensions of the virus in 20 hours. A solution containing 0.5 mg per ml inactivates 100 MLD of vaccinia virus in 3 hours at 37° C and 1000 MLD in 24 hours (Kligler and Bernkopf, 1937).

*Chloroform*.—An excess of chloroform under conditions of constant agitation has been reported to destroy infectivity of vaccinia virus in 60 minutes at 13° C (Yaoi and Kasai, 1931).

*Digestive Enzymes*.—Several enzymes have been tested for their action on viruses. The results of such experiments are strongly influenced by the amount of substrate present other than the virus under investigation. Even when the virus is susceptible to the action of the enzyme, this may not be detectable if the virus repre-

sents only a minute portion of the total susceptible material present. Thus Merrill (1936) was unable to detect inactivation of vaccinia virus by trypsin in the presence of tissue extract, though this enzyme caused a measurable amount of inactivation of washed vaccinia virus. Other workers have concluded from their studies that trypsin has no inactivating action on the viruses of feline pneumonitis, vaccinia and equine encephalomyelitis.

Chymotrypsin has no effect on washed vaccinia elementary bodies or on swine influenza virus, but it does inactivate equine encephalomyelitis and pseudorabies viruses (Merrill, 1936). This enzyme does not inactivate feline pneumonitis virus (Brown, Itatani and Moulder, 1952).

When purified vaccinia virus is treated with papain, it loses its infectivity and characteristic shape with the liberation of amino nitrogen (Hoagland, Ward, Smadel and Rivers, 1940).

Tests for the inactivation of viruses by pepsin are hampered by the fact that the pH at which the enzyme is active rapidly destroys the infectivity of most viruses. Hoagland and his associates, however, found that vaccinia virus is digested by crystalline pepsin at pH 2.

Ribonuclease and desoxyribonuclease do not inactivate viruses even though all viruses studied contain either ribonucleic or desoxyribonucleic acid. Brown, Itatani and Moulder (1952) conclude that this is because the specific chemical groups necessary for enzyme-substrate combination probably are not present on the surface of the elementary bodies.

Lipase rapidly inactivates vaccinia virus (van Rooyen and Rhodes, 1948), but both lipase and lecithinase were found by Brown and his associates to have no action on feline pneumonitis virus.

Treatment of both crude and purified feline pneumonitis virus with trypsin, chymotrypsin or papain raises its infectivity. This is considered by Brown and his associates to be due to dispersion of aggregates of elementary bodies, thus increasing the number of independent infectious units. Tryptic digestion of infected tissue homogenates has been used in the purification of several viruses by the selective digestion of host protein.

From the data available it may be concluded that viruses differ in their susceptibility to various enzymes; but this is clouded by the different degrees of purity of the virus suspensions that have been used. It is obvious that much more work will have to be done before the action of enzymes on viruses is clarified.

*Ethanol.*—The virucidal action of ethyl alcohol is greatly influenced by the presence of substances that are precipitated by alcohol. As chick embryo allantoic fluid contains a small amount of such material, this probably accounts for the seemingly irregular



results obtained in studies on the effect of alcohol on influenza virus suspended in this fluid. When infected allantoic fluid is diluted with physiological saline solution and alcohol is added to make 25 per cent alcohol, the rate of inactivation is slow; but in the presence of 40 per cent alcohol, most of the virus is destroyed in 3 minutes (Dunham and MacNeal, 1944). However, an almost uniform degree of inactivation is caused by higher concentrations of alcohol, 50 to 90 per cent, acting for either 3 or 15 minutes. Titrations show a millionfold destruction of virus under all these conditions, but about 20 per cent of the eggs inoculated with the original mixtures of virus and alcohol become infected with influenza virus (Dunham and Ewing, 1953). A plausible explanation is that an imperceptible precipitate is formed that coats and protects less than one virus particle in a million.

Purified vaccinia virus is itself precipitated by ethyl alcohol. At  $-5^{\circ}\text{C}$ , maximum precipitation is obtained with 25 per cent alcohol as determined by chicken red cell agglutination tests (Cox, van der Scheer, Aiston and Bohnel, 1947).

When a large amount of protein is present, the action of alcohol is greatly diminished. Thus when ethanol is added to make a 75 per cent concentration in blood containing foot and mouth disease virus, the dense coagula that are formed protect the virus from the action of the alcohol (McCulloch, 1945). The addition of as small an amount of sodium hydroxide as 0.005 N, 0.02 per cent, greatly increases the virucidal activity by preventing the formation of a compact precipitate. Under these circumstances, the infectivity of heavily contaminated blood is destroyed in about one minute by 40 per cent ethyl alcohol.

*Ether.*—The viruses differ markedly in their susceptibility to inactivation by diethyl ether. The infectivity of purified influenza virus is destroyed in 90 minutes by a saturated aqueous solution (Stock and Francis, 1940) and the mumps virus in yolk sac emulsion is no longer infective after standing for 30 minutes at  $4^{\circ}\text{C}$  in the presence of 1.5 volumes of ether (Habel, 1945). Members of the psittacosis-lymphogranuloma group have been uniformly reported to be inactivated by diethyl ether. Feline pneumonitis virus is also sensitive to the action of ether. Contact with peroxide-free ether for 15 minutes at  $0^{\circ}\text{C}$  destroys more than 99 per cent of its infectivity (Brown, Itatani and Moulder, 1952).

The poliomyelitis and Coxsackie viruses are relatively resistant to physical and chemical agents, including diethyl ether. For many years the standard method used in the recovery of virus from the stools of poliomyelitis patients was to mix a suspension of the stools with about 15 per cent by volume of ether and to hold in the refrig-

erator for 1 to 3 days. This kills most of the bacteria but sufficient poliomyelitis virus remains to produce infection in monkeys.

Individual variations among the Coxsackie group were sought by Sulkin and Wallis (1952). They found that out of the strains representing 7 types, one was moderately susceptible to this agent. The differences in this regard between several neurotropic viruses were shown by the results of a study in which identical procedures were used for each virus (Sulkin and Zarafonitis, 1947). The lowest concentrations of ether that reduced the viruses 100-fold were in the case of St. Louis encephalitis, 3 per cent; Eastern equine encephalomyelitis, 5 per cent; Western equine encephalomyelitis, 10 per cent; while rabies virus deteriorated less than this in the presence of 30 per cent ether and the Lansing strain of poliomyelitis virus did not undergo any detectable inactivation even in the presence of 95 per cent ether.

*Ethylene Oxide.*—That this relatively simple chemical compound,  $(CH_2)_2O$ , is active against numerous viruses was shown by Ginsberg and Wilson (1950). They employed the agent in the liquid state, performing all manipulations in the cold as the boiling point is  $10.7^\circ C$ . The virus suspensions were exposed to 1 per cent ethylene oxide for 1 hour at  $4^\circ C$  followed by 24 hours at  $35^\circ C$  in containers with cotton plugs. During this period the agent left the mixtures by volatilization. This procedure destroys the infectivity of strong suspensions of such diverse viruses as influenza A and B, Newcastle, mouse encephalomyelitis and vaccinia.

Certain precautions have to be taken in its use. Ethylene oxide is toxic for man, the maximum safe concentration being 100 ppm, though 3,000 ppm may be tolerated for periods up to 60 minutes. Also, it forms explosive mixtures with air and so care must be taken in handling it to prevent accumulation of the vapor in a refrigerator, incubator or room.

*Formaldehyde.*—Formaldehyde is readily available for laboratory use as a solution, formalin, containing 40 gm of the gas in 100 ml of solution. The specific gravity is slightly greater than water so that the formaldehyde represents 37 per cent of the weight. Care should be exercised to distinguish between final concentrations of formaldehyde and dilutions of this standard solution.

Formaldehyde has been used extensively to inactivate viruses in the preparation of vaccines as it has little effect on the antigenic properties. Generally, from 0.2 to 0.4 per cent formalin has been used for this purpose. Because of the irritating properties of formaldehyde, the lowest safe concentration has been sought. Thus Schwerdt, Dick, Herriott and Howe (1951) found that a relatively pure suspension of Lansing poliomyelitis virus with a titer of

10<sup>4.2</sup> retained its infectivity for 12 hours when exposed to 0.1 per cent formalin, but that at 24 hours it no longer produced infections.

Salk (1953) has used formaldehyde for the inactivation of poliomyelitis virus in the preparation of vaccines. He neutralized the normal acidity of formalin by the addition of an excess of solid magnesium carbonate. This solution was added to clear suspensions of virus to produce a 0.4 per cent solution of formalin. The mixtures were kept at 1° C and tested periodically for infectivity. The 3 strains used varied in the time required for inactivation, but this may have been due to differences in the concentrations of the virus in the suspensions. At the end of this period, the action of formaldehyde was arrested by the addition of sodium bisulfite and neutralization of the resulting acid with sodium hydroxide. Another procedure for inactivating the virus is the addition of small amounts of formalin daily for several days to the suspension kept at about 0° C.

The destruction of infectivity of influenza A virus was shown by Lauffer and Wheatley (1949) to be a reaction of the first order, following a logarithmic course. The rate of inactivation decreases as the pH is lowered from 8.0 to 5.6. Below pH 5.6, which is close to the iso-electric point of the virus, the rate is not influenced by the presence of formaldehyde, but is the normal rate characteristic of each temperature and pH value.

*Glycols.*—Triethylene and propylene glycols are active virucides in solution, as aerosols and vapor. However, the physical conditions under which this action takes place have certain specific limits. In aqueous solution, 90 per cent propylene glycol causes rapid inactivation so that a strong suspension of influenza virus is no longer infectious after 3 minutes. An 80 per cent solution inactivates most of the virus in this period, but a 70 per cent solution permits a significant amount to remain active. Vaccinia virus is apparently slightly more sensitive, as a 70 per cent solution causes inactivation of most of the virus in 3 minutes (Dunham and MacNeal, 1943 and 1944). Influenza virus on microscopic flecks of residue from dried droplets can persist in the air for several hours. Triethylene glycol or propylene glycol either atomized or evaporated into air containing such particles rapidly inactivates the virus (Robertson, Loosli, Puck, Bigg and Miller, 1941). Of the two agents, triethylene glycol is effective at much lower concentrations of vapor. The action takes place by the vapor phase, an aerosol serving as a source for the rapid production of vapor (Puck, 1947). The glycol molecules in the air are taken up by the small amount of moisture in the dried droplets of infected material, rapidly producing a virucidal concentration. Because of this condensation, less than 1 ppm in the air is effective. Under experimental conditions, 1 gm of triethylene



glycol in 200,000 L of air, 0.7 ppm, inactivates influenza virus in a few seconds (Robertson, Puck, Lemon and Loosli, 1943). The greatest activity against viruses has been found in the comparatively narrow range of relative humidity extending from about 45 to 65 per cent. By analogy with other experiments, however, it may be that when particles bearing viruses have become unusually dry, the optimum humidity for inactivation with glycol is considerably lower (Robertson and Lester, 1951).

A physical factor that lowers the effectiveness of glycol vapor is the speed with which it leaves the air. Just as the glycol condenses on the particles in the air, so it also is taken up by all moist surfaces including the skin of persons in the room. This can be balanced only by a source of vapor regulated to maintain a constant percentage of saturation of the air. A desirable feature of these agents is their capacity for uniform dissemination throughout all parts of an enclosed space.

*Liquor Antisepticus.*—*Liquor antisepticus* is a mild antiseptic that is extensively used as a mouth wash. The composition of the official preparation has remained essentially the same for decades, though slight changes have been made in the formula from time to time. As defined by the National Formulary, Edition VII, it contains boric acid, 25 g; thymol, 0.5 g; chlorthymol, 0.5 g; menthol, 0.5 g; eucalyptol, 0.1 ml; methyl salicylate, 0.2 ml; oil of thyme, 0.01 ml; ethyl alcohol, 95 per cent, 300 ml; distilled water, q.s. ad 1000 ml.

When this preparation is diluted to 80 per cent strength it rapidly inactivates influenza virus, destroying the infectivity of a strong suspension in more than 30 seconds, but less than 3 minutes. However, full strength *liquor antisepticus* causes loss of infectivity in 30 seconds (Dunham and MacNeal, 1944). Alcohol in the strength contained in this preparation, 28.5 per cent, does not have a comparable action, as mentioned in a previous section.

*Methanol.*—Methyl alcohol produces less denaturation of protein than ethanol. Studies have been conducted by Cox, van der Scheer, Aiston and Bohnel (1947) on the precipitation of influenza viruses A and B from infected allantoic fluid. They found that at 4° C the Weiss and Lee strains are completely precipitated by 31 per cent methanol and that no loss of infectivity occurs even though the virus is kept in contact with the methyl alcohol for as long as 10 hours.

*Mustards.*—The sulfur and nitrogen mustards are of wide interest because of their ability to react with numerous biological compounds *in vivo*. They also have the property of rapidly inactivating viruses. This is true of such diverse viruses as those of

influenza, hog cholera, Eastern and Western equine encephalomyelitis and rabies. The rates of inactivation of viruses are faster than those of enzymes (Herriott, 1948). Sulfur mustard in concentrations as low as  $5 \times 10^{-4}$  M decreases in 30 minutes at  $25^{\circ}$  C the infectivity of influenza virus for chick embryos to 1/1000 of the original and the toxicity for mice by 75 per cent (Fong and Bernal, 1953). The presence of other biological substances in the mixtures affects the action of these compounds on viruses as has been shown to be the case with other virucidal agents. Thus nitrogen mustard

TABLE 60.—DESTRUCTION OF INFECTIVITY OF VIRUS SUSPENSIONS BY SURFACE ACTIVE AGENTS. (FROM DATA BY BURNET AND LUSH.)

*Lowest concentration effective in 2 hours at  $36^{\circ}$  C.*

<i>Virus</i>	<i>Sodium desoxycholate per cent</i>	<i>Saponin per cent</i>	<i>Sodium lauryl sulfate per cent</i>
Herpes	0.05	0.002	0.005
Pseudorabies	0.1	0.005	
Couping Ill	0.1	0.01	0.01
Influenza A	0.2	0.01	0.02
Newcastle Disease	0.2	0.02	
Rabin's B	0.1	0.02	
Myxoma	0.1	Partial, 0.2	0.05
Cowpox	0.2	Partial, 0.2	
Raccinia	0.5	Partial, 0.2	0.05
Cetromelia	1.0	Partial, 0.2	
Sittacosis	Imperceptible, 1.0	Imperceptible, 0.2	Partial, 0.2
Hemolysis	0.1	0.002	0.01

\* See text.

in blood does not readily inactivate the viruses of serum hepatitis and Western and Eastern equine encephalomyelitis, though other viruses under the same conditions are inactivated to the point of extinction of infectivity (Drake, Hampil, Pennel, Spizizen, Henle and Stokes, 1952). In a study on viruses of dissimilar chemical composition, Herriott (1948) showed that mustard gas inactivates those containing desoxyribose nucleic acid more rapidly than the viruses containing ribose nucleic acid. However, the wide range of compounds with which the mustards react has limited their usefulness in elucidating the basic problems of the chemical and other characteristics of viruses.

*Phenol.*—Phenol inactivates viruses, but the concentration required to destroy infectivity depends upon the virus and upon the nature and amount of other substances present. Numerous experiments have been conducted with the agent under a wide variety of conditions. In most instances they indicate that in concentrations below 1 per cent inactivation is either slow or imper-

ceptible. A 1 per cent solution has been used extensively to inactivate rabies virus in the preparation of a vaccine by allowing the mixture to stand for 24 hours at 37° C. A 0.5 per cent concentration does not destroy the infectivity of serum suspensions of the viruses of either equine infectious anemia in 30 days (Stein and Gates, 1952) or Rift Valley fever in 6 months. However, yellow fever virus in serum loses its infectivity in 30 minutes at 30° C when mixed with 0.3 per cent phenol and 6.7 per cent ethyl alcohol.

*Salicylates.*—The concentration of potassium salicylate required to destroy the infectivity of suspensions of several viruses under identical conditions was investigated by Cooke and Best (1941). They found that this was achieved in 24 hours at 22° C by 0.15 M potassium salicylate with the herpes virus, but that 0.3 M strength was required for the Shope papilloma and vaccinia viruses. These studies were extended to determine the rate of inactivation of the Shope papilloma virus in the presence of a constant concentration of potassium salicylate. They found the highly significant fact that the rate of inactivation of the virus is logarithmic. In each interval of time the same fraction of the remaining virus is inactivated.

*Surface Active Agents.*—Fundamental knowledge as to the action of surface active agents on viruses has been limited by the fact that most investigations have been directed to the question of whether infectivity can be destroyed. This type of investigation does not reveal the kinetics of the interactions of viruses and surface active agents. It does, however, indicate the relative activity of of different compounds.

The action of sodium desoxycholate, saponin and sodium dodecyl sulfate on a relatively large number of viruses was studied by Burnet and Lush (1940). They used a single set of experimental conditions, various concentrations of the agents acting for 2 hours at 36° C. Attempts were made to use a comparable number of initial infective units of virus in all experiments. The wide range of susceptibility of the viruses to these agents is shown by the summary given in Table 60. Included in the table are the lowest concentrations of the agents that cause hemolysis of 1 per cent suspensions of rabbit erythrocytes. There is a close relation between these levels and those that are effective against the more susceptible viruses.

The sodium salts of oleic, linoleic and linolenic acids were found by Stock and Francis (1940) to inactivate influenza virus at pH 7.5. Inactivation of feline pneumonitis virus is accomplished rapidly by the cationic surface active agent, cetyl pyridinium chloride, and more slowly by the anionic agent, sodium dodecyl sulfate (Brown, Itatani and Moulder, 1952).



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## 19

# SILVER COMPOUNDS\*

### INTRODUCTION

SILVER is a prehistoric element. It was well known to the ancient Babylonians and the ancient Greeks possessed many silver mines, but there is no definite record of the use of silver compounds in medicine until the eighth century A.D. and their value as local antibacterial agents was not recognized until much later. In the latter part of the nineteenth century, Karl Siegmund Franz Credé (1884) introduced the use of silver nitrate for the prevention of ophthalmia neonatorum. About that time Raulin (1869), von Behring (1890) and von Nägeli (1893) were studying the effects of small quantities of silver and silver nitrate on bacteria and molds. Von Nägeli coined the term "oligodynamic" to indicate such activity. This effect is discussed at length in the following chapter on oligodynamic metals. Later, about 1897, Benno C. Credé reported the use of "Credé's Antiseptic," powdered silver citrate and "Credé's Ointment," colloidal silver incorporated in an ointment base. Both of these materials are used for the treatment of wounds and skin diseases.

The use of silver compounds in medicine is not limited to their antibacterial effects. Although these compounds cannot be absorbed in amounts sufficient to have any action whatsoever, they have been used repeatedly for the treatment of all forms of nervous diseases (insanity, epilepsy, etc.) with uniformly negative results. This therapy was a survival of the fantastic teaching of the middle ages and was based on the dedication of silver to the moon and the supposed connection of the latter with lunacy (Sollmann, 1943). Silver nitrate, and to a lesser extent silver picrate, are used to secure caustic and astringent effects. Concentrated solutions are caustic and weaker ones astringent. The irritation, pain, astringency and corrosion of these compounds may be desirable for the cauterization

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\*Aided by a grant from the Lily-Tulip Cup Corporation.

f wounds, the removal of granulation tissue, warts, etc., but when these effects are not necessary they can be avoided by the use of dilute solutions or colloidal preparations.

## SIMPLE SILVER SALTS

**SILVER NITRATE.**—A few drops of a 1 per cent solution of silver nitrate is routinely instilled in infants' eyes immediately after birth for the prophylaxis of ophthalmia neonatorum. At the present time the sanitary codes of most states require this treatment for all newborn infants. However, because of the chemical conjunctivitis that may result from such treatment, extensive investigations are being made to find a substitute that will be just as effective and less harmful to infants. The indications are that penicillin, by muscular injection or local application as ointment, may be just as effective and is tolerated better by infants. Silver nitrate still is the method of choice, but many sanitary codes, including that of the City of New York, have been modified to include the use of equally effective agents (Greenberg, 1953).

Veterinarians use silver nitrate solutions for the treatment oforrhoea in dogs and sinusitis in turkeys (Merck Index, 1952).

*Toughened Silver Nitrate, U.S.P.* (Lunar Caustic, Silver Nitrate Pencils, Fused Silver Nitrate, etc.) contains not less than 4.5 per cent silver nitrate and 5 per cent silver chloride. The caustic action of these "pencils" is employed in the treatment of small wounds. The "pencil" is touched to the area. One application usually relieves pain and expedites healing.

*Ammoniacal Silver Nitrate Solution, N.F.* (silver diamino nitrate) containing 30.5 per cent silver is used with reducing agents, such as 10 per cent formaldehyde or eugenol to deposit fine particles of silver in infected areas. It is used in dentistry as an antibacterial agent for the treatment of pulp canals of teeth and in controlling dental caries.

**SILVER LACTATE.**—Silver lactate N.N.R. is a white, crystalline powder that is employed for the same purposes as silver nitrate, usually in dilutions varying from 1:100 to 1:2,000. The powder is irritating to wounds.

**SILVER PICRATE.**—Silver picrate N.N.R. is a yellow, crystalline powder which is but sparingly soluble in water and alcohol and slightly soluble in organic solvents. It is used in the treatment of vaginitis, especially trichomonal, monilial and gonorrheal. It is most effectively administered in powder form by insufflation with supplementary application in vaginal suppositories (Goodman and Gilman, 1943).

## COLLOIDAL SILVER PREPARATIONS

Colloidal preparations contain silver or insoluble silver compounds, such as silver oxide, silver halogens or silver protein precipitates in very finely divided particles, some of them ultramicroscopic. They are so finely divided that they appear to go into solution but are actually permanent suspensions of insoluble substances. They are sparingly ionized and, therefore, are relatively non-corrosive, non-astringent and non-irritant. Their solutions do not form visible precipitates of chlorides or proteins and they retain much of their antibacterial activity in the presence of these substances. They are frequently employed for the treatment of mucous membranes in upper respiratory infections, conjunctivitis due to a variety of organisms and the prophylaxis and treatment of gonorrhea and cystitis. Gonorrheal infections are highly susceptible to this type of silver preparation and its greatest use is in the prophylaxis and treatment of gonorrheal conjunctivitis and gonorrheal urethritis. These compounds are used also in colonic irrigations for the treatment of bacillary dysentery.

**SILVER PROTEIN PRECIPITATES.**—Most of the commercial preparations are made by dissolving reduced silver or silver compounds in an excess of denatured protein and drying in vacuo. Substances so prepared disperse freely although somewhat slowly in water, yielding brown "colloidal solutions." The adjectives "strong" and "mild" applied to these compounds refer to therapeutic action, *i.e.*, to the silver ions, and not to the total silver content, which is usually in the reverse order.

*Strong Protein Silver, N.F.* (strong protargin).—Compounds of this type contain the lowest proportion of silver (7.5 to 8.5 per cent). Therapeutically they are intermediate between silver nitrate and mild silver protein. They have the strongest germicidal action of the compounds in this group and are distinctly irritating.

*Mild Silver Protein, U.S.P.* (mild protargin, Argyrol, Argyn, N.N.R. and Silvol, N.N.R.).—These compounds contain from 19 to 25 per cent silver but are non-irritant.

*Collargol* (Argentum Credé).—These compounds contain a higher concentration of silver (78 per cent) in the form of metallic silver reduced to the colloidal form by chemical means and stabilized by alkali albuminate. They are used mainly for intravenous and intramuscular injection.

The concentrations of protein silver compounds used on mucous membranes range from 0.1 to 10 per cent for strong silver protein, from 5 to 50 per cent for mild silver protein and from 0.02 to 1 per cent for collargol. These act best when applied every two hours.



For antiseptic purposes the solutions should be freshly prepared and should be protected from light. Ointments and suppositories are used with the same concentrations as the aqueous solutions. The usual concentrations for special purposes are shown in Table 61.

TABLE 61.—THERAPEUTIC USES AND DOSES OF PROTEIN SILVER (N.N.R., 1942).

	<i>Strong protein silver per cent</i>	<i>Mild protein silver per cent</i>
Eye:		
Conjunctivitis, simple, purulent or gonorrheal	2 to 10	Solution, 25 Ointment, 10
Prophylaxis against ophthalmia neonatorum	2 to 10	25
Prophylaxis before ophthalmic operations (several days)		25
Corneal ulcers		50
Nose and throat	0.5 to 10	Spray, 10 to 20 Swab, 25 to 50
Wounds and ulcers		1 to 10, solution or ointment 10, dusting powder
Gonorrhea:		
Injections—prophylactic	2	10
Acute	$\frac{1}{4}$ to 1	3 to 10
Chronic	2 to 10	10 to 20
Urethral irrigation	1:2,000 to 1:1,000	1:1,000
Urethral suppositories	5 to 10	20 (0.13 g or 2 grains)
Cystitis		20 to 50 (5 ml) or 10 to 25 (30 ml) left in the bladder
Gynecologic practice		
Solutions	2 to 10	25 (tampons of solution in glycerin)
Tampons	2	
Ointments	5	
Suppositories	5	Suppositories, 20 (0.3 g or 5 grains)
Rectal administration:		
Irrigation	0.1	0.1 to 1
Injection	2	10
Suppositories	5 to 10	20 (0.13 g or 2 grains)
Pyelography		2 (solargentum) 50 (cargentos)

Two per cent strong silver solution is used by injection for early preventive treatment of venereal diseases. Its efficacy is marked if a thorough treatment is applied within one hour after exposure, and is fair up to three hours (New and Nonofficial Remedies, 1952). A solution of from 2 to 10 per cent is used in the eye. A 25 per cent solution of mild silver protein solution is employed

in conjunctivitis of a simple purulent or gonorrheal origin. Solutions of from 10 to 25 per cent are used in the nose and throat, on wounds and ulcers, and 10 to 85 per cent solutions are used as a prophylactic injection in gonorrhea (Krantz and Carr, 1949). Sollmann (1943) thought that compounds of this group act largely as mucilaginous demulcents and protectives, and as detergents by dislodging pus.

**COLLOIDAL SILVER HALOGENS.** — *Colloidal Silver Chloride, N.F.* (Lunosol, N.N.R.) is silver chloride rendered colloidal by sucrose and a suitable protective colloid. It contains from 9 to 11 per cent silver chloride. Solutions are opalescent suspensions having a reddish-blue hue.

*Colloidal Silver Iodide, N.F.* (Neo-Silvol, N.N.R.) contains approximately 20 per cent silver iodide in a gelatin base. It forms colorless, milky or opalescent solutions in concentrations up to 50 per cent in water.

Neither silver iodide nor silver chloride precipitate protein or irritate tissues, and both are probably germicidal only in high concentrations. They do not stain the skin but are affected by light. They are generally used in from 5 to 25 per cent solutions but 10 to 40 per cent solutions are used in inflammatory infections in the eye, ear, nose and throat. These solutions are used for the treatment of infections of mucous membranes of the genito-urinary tract and for prophylaxis against such infections.

**COLLOIDAL SILVER OXIDE.**—Müller (1937) suspended colloidal silver oxide, particle size less than 0.5 micron, in paraffin hydrocarbons to produce a fluid or semifluid therapeutic compound. A 5 per cent suspension of this silver oxide in mineral oil can be injected through the teat canal into udders infected with *Streptococcus agalactiae* for the control of bovine mastitis. An ointment containing 5 per cent of this oxide in a petrolatum base is used as a bacteriostatic agent in infected lesions, such as cuts and abrasions of the skin and mucous membranes and in eye infections. Capsules containing 5 per cent silver oxide and 2 per cent cupric oxide are used for the control of white scours (infectious diarrhea, acute dysentery, etc.) in young or newborn calves.

### MECHANISM OF ACTION

Müller (1937) thought the exceedingly small particle size of the colloidal silver oxide in his preparations enabled it to pass through the normal skin or pores promptly reaching more watery tissue and entering the blood stream as a hydroxide or other form of ionic silver solution. Thus, it could reach infected regions of the body not ordinarily accessible by superficial application of a medicinal compound.

Goodman and Gilman (1943) thought that the toxic effect of silver compounds on microorganisms is due to the silver ions which precipitate the protein of bacterial protoplasm. The silver proteinate so formed contributes to a sustained antiseptic action by slowly liberating small amounts of silver ions. They thought it probable that in the use of simple salts of silver as antiseptics some metallic silver is obtained by reduction and that its oligodynamic action contributes to the bacteriostatic effect.

TABLE 62.—KILLING CONCENTRATIONS FOR TISSUE AND BACTERIA AND CORRESPONDING TOXICITY INDICES AND PHENOL COEFFICIENTS IN TEN MINUTES AT 37° C. (SALLE, McOMIE, SCHECHMEISTER, AND FOORD, 1939.)

Compound	Tissue (A)	Killing dilution		Toxicity index = A/B		Phenol coefficient	
		<i>M. pyogenes</i> var. <i>aureus</i> (B)	<i>S. ty-</i> <i>phosa</i> (B)	<i>M. pyogenes</i> var. <i>aureus</i>	<i>S. ty-</i> <i>phosa</i>	<i>M. pyogenes</i> var. <i>aureus</i>	<i>S. ty-</i> <i>phosa</i>
Lactate	1:100	1:110	1:690	0.9	0.15	1.0	3.7
Citrate	1:610	1:600	1:4500	1.0	0.14	5.5	24.2
Nitrate	1:140	1:80	1:1250	1.8	0.11	0.7	6.7
Protein strong, pH 7.0	1:25	1:15	1:175	1.7	0.14	0.14	0.94
Protein mild, pH 7.0	1:30	1:12*	1:70	2.5+	0.43	0.11	0.38

\* Failed to kill. A more concentrated solution could not be prepared. All results are the average of three determinations.

Sollmann (1943) described the action of silver nitrate in two stages: "(1) the immediate, irritant and germicidal effects produced by the direct application of the free silver ions; and (2) the later, milder antiseptic effects produced by the re-solution and ionization of the silver-protein compounds that were formed in the first stage." He thought that the direct application of colloidal silver proteinates might have advantages over their indirect production from silver nitrate. They cause no irritation; the absence of coagulation membrane would facilitate their access to the cells; they form more concentrated solutions than are likely to be formed from the reduction of the silver precipitates in situ; the colloidal aggregates are likely to be smaller and, therefore, more active; and because of the absence of irritation, they can be applied more frequently and, therefore, secure a more continuous action.

## TOXICOLOGY OF SILVER

The toxicity index (ratio of highest dilution of germicide required to prevent the growth of embryonic tissue in forty-eight hours to the highest dilution required to kill the test organism in ten minutes) of various silver compounds was determined by Salle, McOmie, Schechmeister and Foord (1939). Their results are shown in Table 62. Theoretically an index less than one means that the



germicide is more toxic to bacteria than to embryonic tissue. Therefore, the smaller the toxicity index the more nearly perfect the germicide. All the silver compounds tested were more toxic for *Salmonella typhosa* but were less or just as toxic for *Micrococcus pyogenes* var. *aureus* than they are for embryonic tissue.

New and Nonofficial Remedies cautions that "the long continued use of any silver preparation may produce irremediable discoloration of the skin or mucous membranes (argyria)."

Krantz and Carr (1949) state that silver salts do not produce acute systemic poisoning even when large doses are taken by mouth. They thought that the precipitation of the metal ion by chlorides and by proteins prevents the absorption of a sufficient amount to produce acute generalized poisoning.

However, the long continued use of a silver preparation may produce chronic intoxication, and a bluish-black discoloration of the skin, known as argyria. They thought that this condition probably resulted from the deposition of organo-silver compounds in the skin, where the action of sunlight reduces the silver salts to the dark colored compounds. Argyria persists indefinitely, and there is no known treatment.

After a review and evaluation of cases of generalized argyria reported in the medical literature in the past decade Hill and Pillsbury (1939) concluded that argyria is a permanent, often highly disfiguring change, but produces no significant disturbance of the physiology of the affected organs, or of the general health of the patient. The available evidence indicated to them that as much as 6 to 8 g silver arsphenamine may be given intravenously, up to 6 g silver nitrate may be given in divided doses by mouth, or nasal instillation of the silver salt may be used daily for a period of three months without producing pigmentation of the skin. Silver oxide is the only silver compound that has not been reported as a cause of argyria. However, they think that experience in human subjects has not been extensive enough to be sure that it will not cause discoloration.

Sollmann (1943) observed that silver nitrate in doses of 0.01 to 0.1 g by mouth produces no symptoms and swallowing pieces of pencils up to 2.5 g is often harmless, but larger quantities cause acute gastritis. These reactions are purely local. From 2 to 30 g has caused death within a few hours to a few days; 10 g are generally fatal, but the ingestion of 30 g has been survived.

## SUMMARY

For many years silver compounds were considered the most effective agents available for the prevention and treatment of

gonorrheal infections. This is shown by the almost universal use of silver nitrate for prophylaxis of ophthalmia neonatorum and the use of the silver proteinates for prophylaxis of gonorrheal diseases. The silver proteinates, especially of the argyrol type, have been used extensively in the treatment of infections of the mucous membranes of the eyes, ears, nose and throat. Thus it has been shown that silver compounds are useful germicides and that effective doses are harmless.

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## 20

# OLIGODYNAMIC METALS

## INTRODUCTION

THE consensus of those who have studied the oligodynamic action of metals on biological processes is very much like that of the blind men who studied the elephant in the old Buddhist parable translated by Burlingame (1923). After feeling different portions of the elephant each group reported on what an elephant was like. Those who felt the head said that it was like a water pot; those who felt the ears said that it was like a winnowing basket; those who felt the tusks said that it was like a plowshare, etc. Although each studied only a small portion of the elephant they argued that the remainder was the same and any opinion to the contrary was wrong. Many investigators who have studied oligodynamic silver, for instance, have not always taken into consideration the several valences possible for silver, the various complexes that it can form, the effect of other metals or the effect of adsorbent, oxidizing or reducing materials on silver and silver compounds and so have reported theories and reactions that are disconcertingly analogous to the reports of the blind men described by Gautama Buddha.

The term "oligodynamic" was first used by von Nægeli (1893) to differentiate between the effect of silver nitrate on living matter in concentrations of 10 ppm, "ordinary" or "poisoning" death and greater dilutions, "oligodynamic" death. Thus, he combined the Greek words "oligos" meaning small and "dynamis" meaning power to form a word meaning effective in small quantities, and this meaning has been generally accepted. Berk (1947), in his excellent review of the literature on antibacterial activity of oligodynamic metals, pointed out that this action was observed earlier and was reported by Raulin (1869), von Behring (1890) and Uffelmann (1892). However, von Nægeli coined the word and was first to observe the ability of silver solutions to activate other material, in this instance, by adsorption on glass walls of the container.

\*Aided by a grant from the Lily-Tulip Cup Corporation.  
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## THEORIES REGARDING THE MECHANISM AND EFFECT OF OLIGODYNAMIC ACTIVITY

*Solution of Metals.*—Von Behring (1890) appears to be the first to have advanced a theory as to the mechanism of oligodynamic action. He concluded that the activity of metals on inoculated agar plates was caused by dissolution of metal, and the variation in activity was caused by variations in solubility of metals in different bacterial waste products. This was confirmed by Credé (1901) while studying the effect of silver leaf in inoculated cultures and infected wounds, and by Löhner (1919) and Doerr (1921). Saxl (1917*d*) thought that his studies on the preparation of sterile vaccines that showed equal activity of insoluble mercurous chloride and slightly soluble mercuric chloride was evidence against this theory. Other investigators, Ficker (1898), Natonek and Reitmann (1915), Schlossberger (1918), Köhler (1919), Acél (1920), Luger (1920), Sordelli and Wernicke (1921) and Fischer (1924) believed that oligodynamy of metals was essentially simple chemical solution and diffusion. Falta and Richter-Quittner (1921), Herzberg (1923) and Degkwitz (1929) thought that the oligodynamic effect was due to chemical solution of or catalysis by metals.

*Effect on Cell Structure.*—The first attempt to describe the effect of metals on cell structure was made by Israel and Klingmann (1897). Investigating the effect on *Spirogyra*, they described "plasmochism" or separation of two plasma membranes caused by oligodynamic action and plasmolysis caused by poisoning. Plasmochism results when copper penetrates the outer cell wall causing protoplasm threads and chlorophyll bands to contract; threads tear and a small amount of copper penetrates plasma membranes where it goes into chemical combination. Further diffusion of copper into cell interior can cause death without visible change in the cell substance. Galeotti (1901) observed the same effect and believed it to be merely preliminary to plasmolysis.

While studying the effect of silver chloride on bacteria under the electron microscope Kliewe, Steyskal and Steyskal (1947) observed that silver chloride particles always adhered to the bacteria, the bacterial membrane appeared faded, the substance drawn together and the body of the bacterium was lighter. After 2 days contact silver chloride hydrosol caused complete disintegration of anthrax bacilli; the surface of the organism appeared faded and worn. The cytoplasm had become lighter or had gone completely into solution.

*Telergy.*—Blondlot (1904) observed "N" rays that are totally different from X-rays issuing from upper surface of silver metal held in flame 45 degrees to horizontal and reported that wave lengths ran from 30A to 80A.

As a result of his studies of the oligodynamic effects of metals, especially Ag, Cu, Hg and metal salts, Saxl (1917 *a, b, c*) (1919) concluded that oligodynamic action was caused by a physical energy manifesting itself on the surface of metals and that this energy could be separated from the parent metal. He called this action at a distance "Fernwirkung," and Berk (1947) translated it "Telergy." Saxl believed this force could be conducted by platinum wire or silk thread, permeated through rubber membrane as well as into glass, and could activate other bodies. Other investigators, Baumgarten and Luger (1918), Spät (1920), Doerr (1920*b*), Weltman (1920), Laubenheimer (1921), Schnabel (1922), Süpfle (1922) and Bührmann (1933) were able to confirm Saxl's results using mercurous and mercuric chloride but demonstrated that such oligodynamic action was due to volatilization, decomposition or formation of difficultly soluble compounds. Weltman, Laubenheimer and Schlossberger were unable to confirm Saxl's results using other metals and their salts.

Nadson and Stern (1933) were able to inhibit growth of various bacteria and yeasts on inoculated plates for 2 to 3 days by suspending various metals 1 mm above media surface. They felt that this telergy was caused by radio activity of ambient, causing metal to produce secondary radiations of considerable bactericidal power.

*Adsorption by Bacteria.*—Bechhold (1909), studying disinfection from the standpoint of colloidal chemistry, concluded that bacterial growth and death follow adsorption and that chemical combination for poisoning occurs after adsorption by the organism's surface. Morawitz (1910) demonstrated that anions influenced adsorption and that is the first stage in disinfection.

Spiro (1915) studied the effect of copper on yeast cells and concluded that adsorption is the basis of oligodynamic action. Later (1916) he studied adsorption of metal ions by bacteria and concluded that such adsorption on cell surface is the preliminary stage to a subsequent chemical reaction of metal ion and cell structure. When Schlossberger (1918) was unable to confirm Saxl's theory of radiation he concluded that oligodynamic action depended on adsorption of small quantities of metals from solution by surfaces of bacteria. Von Plotho (1920) found the absorption and adsorption of colloidal metals were conditioned by the quality of electric charges on the organism's surface, in substrate and on colloidal particles.

Drechsel (1921), Pichler and Wörber (1922) and Süpfle (1922) investigated the effect of metals and metallic salts on *Spirogyra*, spores and bacteria. They concluded that, in general, adsorption

depends on the dissolution and diffusion of oligodynamically active material, the production of free metallic ions and the amount of adsorbing surface available. Süpfle observed that adsorption by bacterial surfaces concentrated the dissolved metal salts on them, making weak solutions effective and causing death of the cell.

Leitner (1930) working with *Escherichia coli* suspensions and silver and copper salts, tested the effect of various other salts on oligodynamic activity and adsorption of metal ions by bacterial cells. He concluded that some reduce number of free metal ions available for such activity by formation of metal complex salts. Other salts, such as sodium chloride, calcium chloride and magnesium sulfate decreased bactericidal activity by lowering negative charge on bacteria, thus reducing their power to adsorb metal ions. Alkalies increase negative charge on bacteria, promoting their adsorption of metal ions and increasing bactericidal activity.

Von Neergaard (1930) had demonstrated previously that bactericidal efficiency of silver nitrate is not to be evaluated by its concentration of silver salt but that it depends primarily upon its relation to amount of bacterial surface available and its adsorptive power.

Bancroft and Richter (1931) demonstrated that disinfectant action due to heavy metals such as silver was a result of adsorption of metal ions causing coagulation of colloids of the protoplasm. Low concentration caused reversible coagulation (bacteriostasis) and higher concentrations irreversible coagulation (death).

Schwartz and Steinhart (1933) concluded from their work with *Aspergillus niger* that removable copper is held by adsorption and the primary changes are probably in protoplasmic membranes where they thought the change was one of permeability.

Fleming and Young (1940) observed that sensitive strains of coliform bacteria became more resistant to tellurite when in presence of resistant strains and, therefore, thought that resistant bacteria adsorbed enough metal to reduce content in medium to a point where it became harmless to sensitive bacteria.

Of the bacteria studied by Jakob and Mahl (1948) only one strain of *Clostridium tetani* adsorbed silver and gold colloids and then only on the flagella, probably because flagella and the cell body carry different metabolic products and so are not uniformly charged.

*Adsorption of Bacteria.*—Bechhold (1925a) investigated adsorption of bacteria by various substances and observed that adsorption increased with the fineness of the substance and that proportionality disappeared when the particle size approached that of bacteria (1 to 2 $\mu$ ). He developed adsorptive surfaces that not only retained bacteria on their surfaces, but killed them as well. Boluses



and charcoal covered with a thin coat of silver adsorbed more bacteria than did uncoated ones. Renner (1928) confirmed Bechhold's antibacterial results but believed that silicate coated with silver chloride was bactericidal because of its solubility, rather than from adsorption.

*Reaction of Metals with Proteins.*—Paal (1902) pointed out that metal, as hydroxide, is retained or adsorbed by alkaline solutions of protein substances and is, therefore, held in the protein molecule as an ion and not in so-called organic bond. After working with egg or serum albumin and copper sulfate or silver nitrate, Galeotti (1903) thought that the so-called metal albuminate precipitates from mixtures of heavy metal and albumin were loosely bound in varying proportions and could be dissolved in excess of either original compound.

While studying the effect of the copper group metals (Cu, Hg and Ag) on cells of lower plants, Bokorny (1906) found them to be bactericidal in low concentration ( $10^{-7}$ ), and concluded that they react with protoplasmic albumin in sufficient quantities to be injurious. Bohtz (1916) studied the effect of metal powders on infected agar and concluded that they formed metal albuminates, thus becoming antiseptic. Bernhard (1921) observed that bacterial disinfection by heavy metals made combinations with the protoplasmic albumin of the bacterial cell, forming insoluble compounds.

Working with pure solutions of albumin and globulin, von Neergaard (1925 *a, b*) found that silver ions are bound primarily by albumin. Results indicated adsorption rather than chemical binding with proportionately greater adsorption at lower concentrations of silver. He concluded that silver albumin compounds are precipitable, non-reversible and insoluble in an excess of coagulant. He thought that such compounds can form only after Cl was saturated by Ag and the excess Ag had been able to diffuse through the cell membrane. The concentration of disinfectant on bacteria was more important than the concentration in solution.

On the contrary, Gutstein (1927) thought that disinfection is the result of chemical binding and chemical change rather than mere adsorption of metal. After studying locale of action of heavy metals and dyes in bacterial cells by various chemical and staining techniques he concluded that heavy metals always concentrate in protoplasmic membrane and assumed that primarily chemical substances, *i.e.* lipoids containing phosphatides, in the membrane are affected by heavy metal disinfectants.

After a critical review of the work of previous investigators, Neisser and Eichbaum (1932) concluded that oligodynamic metals act through formation of metal protein complexes with living sub-

stances, toxins and enzymes or as catalysts. By electrolysis of horse serum treated with silver, Zittle (1938) concluded that compounds are formed between silver and proteins that cannot be broken down by washing with water or by electrodialysis. Goetz (1943) thought that silver ions adsorbed by cell surfaces form irreversible silver proteinates and thus cause death.

*Effect of a Combination of Metals on Oligodynamic Activity.*—After investigating the oligodynamic effect of single and various combinations of metals on *Micrococcus pyogenes* var. *aureus* in solid agar, Thiele and Wolf (1899) concluded that absolutely pure metals are inactive but that they can be activated by minute amounts of other metals. They studied the effect of metals in combination by placing on the surface of an inoculated agar plate two pieces of dissimilar metals connected with each other by a bow of either metal strip bridging across intervening agar surface. Some combinations showed no effect, others showed considerable increase in oligodynamic action. For instance, silver connected with gold, platinum or lead gave a sterile zone five times larger than from silver alone. They likened this action to that of an electrolytic cell in which the nutrient medium is the electrolyte. Dissimilar metal plates act as electrodes and when connected by a bridge conductor a closed circuit is provided, the direction of current depending on the position of the metals in the electromotive series.

Bitter (1911), Messerschmidt (1916), Löhner (1919) and Affonso (1920) concluded that impurities of other metals are essential for oligodynamic action. Pfeiffer and Kadletz (1917) confirmed the increased activity of paired metals and Saxl (1917*d*) confirmed the booster effect of various metal combinations but could find no support for the electro-potential theory. Bechhold (1918) found that he could increase the bactericidal activity of metals by mixing various metal covered boluses together "thereby obtaining millions of galvanic circuits designated as 'disperse galvanic couples'." Mixtures of colloidal metal were nongalvanic.

Kuroya (1927) observed that the addition of metal powders to metal salts increased their oligodynamic activity. Krause (1928) increased the activity of pure silver by the addition of an activating metal below silver in the electropotential series, such as palladium or gold (Katadyn Silver). Goetz, Tracey and Harris (1940) observed that the addition of another metal does not always increase oligodynamic activity and concluded that the effect was due to their relative position in the electro-potential series.

Guest and Salle (1942) investigated various metallic salts, alone and in combinations, against *M. pyogenes* var. *aureus*. In every case the mixture of two salts, one in higher state of oxidation

than other, resulted in increased germicidal action. Kliewe *et al.* (1947) found no increase in bactericidal activity when various metal aerosols (fine powdered metals dispersed with air) were combined.

*Metallic Salts.*—When Raulin (1869) observed that *A. niger* could not grow in silver vessels he concluded that growth was prevented by minute amounts of metal or metal salts which were formed by chemical action of liquid medium. After studying the germicidal effect of metallic silver, silver oxide and silver nitrate, Messerschmidt (1916) and Doerr (1920 *a, b*) thought the oligodynamic activity of a metal depended on the formation of difficultly soluble metallic salts on its surface which on dissolution in water could produce cytotoxic ions.

Süpfle (1929), using microorganisms that can grow both aerobically and anaerobically, demonstrated that oxygen was not necessary for germicidal activity of silver nitrate. However, colloidal silver (Kallargol) required oxygen for the formation of silver compounds which could subsequently produce silver ions. Silver salts could produce such ions without mediation of oxygen. Murto (1930) believed that the oligodynamic action resulted from the neutralization of positive metal ions by bacteria and that it did not depend on metal salts on metal surfaces.

Neisser (1932) showed the importance of silver salts in the oligodynamic process and Neisser and Eichbaum (1932) thought that, in such processes, surface metal salts must be formed which can subsequently dissociate and ionize. Gibbard (1933*a*) observed that low temperatures had less effect on silver nitrate than on silver ions and that silver nitrate was inactivated by broth, probably because of combination with it.

Goetz *et al.* (1940) concluded that metals become oligodynamically active when an active deposit is produced on their surface. This can be accomplished by chemical or electro-chemical deposition of a compound that dissociates and is soluble. They thought the solubilities of most silver compounds sufficient for germicidal action, with the possible exception of silver sulfide. After studying the killing action of water activated by metallic silver and dilute solutions of silver nitrate on *Paramecium*, Gauze (1940) concluded that both showed the same mechanism of killing the cell.

*Metallic Oxides.*—Spiro (1915) observed that oxygen plays an important part in oligodynamic action and Doerr and Berger (1922) made silver metal oligodynamically active by heating it in a strong air blast for a short time and deactivated it by heating to the melting point. Buschke, Jacobsohn and Klopstock (1926)



thought that the oligodynamic action depended on metal oxidation products formed in the presence of air.

LaCava (1931) demonstrated increased activity on infected agar plates of coins that had been oxidized with high frequency current or immersed in hydrogen peroxide. Pilod and Codville (1932) concluded that metals require oxygen and carbon dioxide to activate water. After investigating different means of making silver metal oligodynamically active Lieb (1936) concluded that oxidizers were the best activators.

Lisbonne and Seigneurin (1936) observed that absence of oxygen inhibited oligodynamic action of mercury in water. Later, Seigneurin and Renoux (1943) concluded that activation of water by mercury was a chemical phenomenon; the mercury was oxidized, and then the compound dissolved in traces.

Goetz *et al.* (1940) observed that clean, oligodynamically inactive silver can be activated by the formation of oxide layers at the surface of the metal. However, Grumbach and Wehrli (1948) observed synergistic action between silver ions and thioglycollate agar producing greater inhibition of the bacteria tested but they did not attempt to explain it.

*Metallic Chlorides.*—After studying the action of silver preparations in dilute sodium chloride solutions, Gros (1911) concluded that such solutions favored finer grained silver precipitates with inherently greater solution surface.

Bernhard (1921) observed that silver has a strong affinity for chlorides, that silver chloride is formed from sodium chloride regularly present in body fluids in the presence of albumin, then the silver chloride formed is peptized by the albumin. Lieb (1935) accepted the theory that oligodynamy was due to metal surface compounds and thought silver chloride best suited for practical use as a germicide.

Kruse and Fischer (1936) thought the active agent in the Electro-Katadyn process was silver chloride formed electrically on silver anode only, assuming that during the process Cl ions were produced from chlorides present in most waters and that they combined with silver ions on the silver anode. They developed the Cuma process, using a silver cathode and a non-reacting anode to produce chlorine from chloride in water, thus producing silver chloride that they believed to be more active than that produced by other methods.

Fromberg and Heiss (1937), using agar plates infected with *pyogenes* var. *aureus*, confirmed the inactivity of clean, pure silver wire, demonstrated that such a wire could be activated by coating one end with silver chloride and that non-active wire could be activated by touching coated wire in an agar plate, causing both

wires to produce a zone of inhibition. When the wires were in separate plates the clean wire could be activated only if the plates were joined by both silver wire and agar bridges, indicating that silver chloride produced an ion which could be transferred to inactive silver by an electropotential difference through a suitably closed electric circuit.

Using colloidal silver chloride, particle size 0.1 to 1.0  $\mu$ , and air, oxygen or other gas as the dispersing agent, Kliewe *et al.* (1947) prepared a silver chloride aerosol. The results of studies of bactericidal effects of this product and hydrosols made with silver chloride or silver prepared in the same manner convinced them that the action of silver and silver chloride aerosols is based not only on ionized silver but also on complex formation.

Cade (1947) demonstrated that the transfer of a silver nitrate treated disc from agar inoculated with *M. pyogenes* var. *aureus* containing no sodium chloride to infected agar containing 2.0 per cent sodium chloride resulted in an increase in the zone of inhibition from 2.5 to 5 mm. A similar effect was obtained when sodium chloride was added to a silver nitrate treated disc. Gesser (1952) increased the antibacterial effect of silver and silver compounds by adding sodium chloride to a solution containing them and electrolyzing it in an iron container (used as anode) with a carbon or graphite cathode. No destruction of bacteria occurred with electrolysis in the absence of silver. Since silver was not connected to the circuit he assumed that it served as an "intermediate conductor."

*Metallic Ions.*—Gros (1911) studied the bactericidal action of silver preparations and concluded that it was a result of the union of the silver ions in solution with a "life-essential part" of the bacterium. Bechhold (1918), Salus (1919), Drechsel (1921), Andresen (1928), Süpfle (1929) and Hämäläinen (1930-31) believed that disinfection with metals and metal salts depended on the number of metal ions in solution. After a thorough study of the action of salts and ions on bacteria, Eisenberg (1919) concluded that toxicity of salts was an additive function of colloid chemical activity of the component ions and that the kind and amount of electric charge on the ions and the ease with which their charges can be transferred was of fundamental importance. Doerr and Berger (1922) believed that oligodynamy was caused by the amount of silver ions or ionizable silver compounds, not by colloidal silver or by separation of silver compounds because such compounds that did not dissociate to give silver ions, but left silver as a part of another compound ion, were very weak disinfectants.

Von Neergaard (1925c) investigated the effect of body electrolytes on the concentration of silver ions. He assumed that albumin acted as the adsorbent and silver as the adsorbate. With the procedure of adsorption, the silver ion concentration is lowered, promoting further dissolution of silver from its source and as the adsorbability of albumin gradually decreases through a saturation of its valences, the chloride ion concentration governs more and more until it finally determines maximum silver concentration.

As a result of their investigation of the effect of copper salts on disinfection and growth, Vignatti and Schnabel (1928) concluded that copper ions had similar effects on bacteria as on solution of lipoids and albumin, namely, a microcoagulation of bacterial colloids, as shown by the increased nephelometric effect. The change occurs in ectoplasma membranes, prevents further diffusion of nutrients from the surrounding medium to the cell interior, produces bacteriostasis and can cause death.

Leitner (1929) tested the effects of silver activated water and silver chloride solution with equal silver ion concentration. Since both showed equal bactericidal activity he assumed that oligodynamic action depended greatly on the metal ion concentration. Vernicke and Modern (1929) observed that the presence of oxygen made no difference to silver or copper already present in solution and so concluded that bactericidal action occurs through the direct action of metal ions and not by the oxidation of bacteria. Murto (1930) believed that oligodynamic bactericidal action occurred because of neutralization of positive metal ions by bacteria. Samaan (1930) observed the effect of silver colloid and silver nitrate on washed *Salmonella typhosa* cells and concluded that the colloid was less effective than ionic silver in silver nitrate. Pilod and Rodville (1932) observed that oligodynamic activity varied directly with the contact time and inversely with dilution of the metal ions. Langwell (1932) concluded that metals can produce toxic ions and that there is a difference in the activity of a metal and its ions.

Neisser (1932) quoted Leitner as stating that after adsorption of silver ions by bacteria  $1/2$  to  $1/5$  of the original silver ions remained in water.

Neisser and Eichbaum (1932) concluded that the dissolution of oligodynamic metal can proceed in the form of free metal ions, complex ions or as colloids, depending upon the electrolytic and organic constituents of the dissolution medium. While Casagrande and Sepilli (1934) were investigating Katadyn jars (containing silver covered bodies) they observed that electrostatic measurements indicated that bacterial suspensions lost some electric charge



after being in contact with silver ions, probably because of fixation of the ions on the bacterial cell.

Just and Szniolis (1936) pointed out that, if the silver dose for water sterilization is 40 gamma/L, the number of silver ions thus provided amounts to  $2.4 \times 10^{11}$ /ml which number is quite sufficient to make contact with any number of bacteria usually present in water. Goetz *et al.* (1940) concluded that only 1 silver ion was required to kill a bacterium but that the high silver adsorptive capacity of bacterial surfaces can shield other cells. Later Goetz (1943) stated that silver is germicidal only if it is in an ionic state and in contact with a cell surface. Rochat and Uzdins (1947) thought that oligodynamic action was due to the presence of metallic ions in water and the selective adsorption of these ions by cellular surfaces. The results of a study of the bactericidal action of some colloidal silver compounds caused Buonomini and Lapucci (1950) to favor the view that oligodynamic action is due to particular ionic conditions.

*Formation of Complex Ions.*—Liese (1924) observed that ammoniacal silver nitrate solutions are much less antibacterial than are silver nitrate solutions. He thought that this was because silver was not in the form of free ion but in a complex,  $\text{Ag}(\text{NH}_3)_2$ .

Von Neergaard (1925a) cited Lottermooser and Mayer to explain the action of silver chloride, stating that an ultramicroscopic silver chloride particle adsorbs an ion on its surface producing a negatively charged complex ion,  $(\text{AgCl})\text{-Cl}^-$  and a free silver ion,  $\text{Ag}^+$ . Conversely, sodium chloride added to silver nitrate forms a positively charged complex ion,  $(\text{AgCl})\text{-Ag}^+$  and a free chloride ion,  $\text{Cl}^-$ . Later (1925b) he observed the formation of Na-Ag-Cl complexes paralleling the binding of silver to albumin, and in equilibrium with each other. He thought that the inhibition of *E. coli* in bouillon by 0.19 gamma Ag per liter, when in distilled water 13 gamma were required for same effect, was a result of formation of Na-Ag-Cl complex in the bouillon because this complex is sufficiently soluble to make it bactericidal also. He (1925c) summarized the process of silver albumin action thus: Silver ions plus sodium chloride of organism form complexes Na-Ag-Cl. When the silver ion concentration becomes small compared with complex-bound silver, they have a tendency to decompose, the albumin pulling the free silver ion to it, which reduces the concentration of free ion solution causing further decomposition of the silver complex. This balancing process continues until the adsorptive capacity of albumin for silver is decreased to such an extent that it can no longer promote decomposition of weakly bound  $\text{AgCl}_2$ . Since it appears that the principal cause of cytotoxic action is the adsorption

of silver by albumin, the power of this action depends on the kind of complex formed. If a strong complex like  $\text{KAg}(\text{CN})_2$  is formed, the inability of silver to split off prevents adsorption and there is no cytotoxic action. Bactericidal action depends greatly on the formation of a stable complex. It is possible that some silver complexes are adsorbed bodily as complex ions by albumin.

After further study, von Neergaard (1925*d*) concluded that the adsorption of silver by bacterial albumin is quantitatively dependent on the silver ion concentration of the solution. The force that promotes adsorption is limited to that with which silver ions seek to cling to inorganic complexes like  $(\text{AgCl})\text{-Ag}$  or  $\text{Na-Ag-Cl}$ . When the adsorptive power of bacterial surfaces is much greater than the force required to maintain silver binding on inorganic complexes, the silver ions are adsorbed, further decomposition of the complexes in solution is promoted and the bacterial surfaces are able to adsorb sufficient silver ions to result in disinfection.

Kliewe *et al.* (1947) thought that the formation of complex compounds, especially  $\text{AgCl}_2$ , in physiological salt solutions was apparent, not so much by an increase in the solubility of silver chloride, but by an enormous decrease of potentiometric activity and so of free silver ions. Only about 1/5000 of the total silver would be in the form of ions. They cited von Neergaard who reported that although silver was bactericidal in bouillon and blood serum, only 1 ppm silver in bouillon and 5 ppm in blood serum was present as ions. They concluded that metals owe their bactericidal action to their tendency to act as control atoms of complex compounds and to bind electro-negative as well as neutral atom groups in their immediate action radius and exchange them under certain circumstances for others. They thought that the formation of a silver ion complex depended on the silver ion concentration and the concentration of all other complex forming ions in the solution. It seemed to them reasonable to conclude that silver ions form complexes with amino-acids and bases which possibly inhibit the development of bacteria.

*Effect on Enzymes.*—Investigation of the effects of metals on fermentation processes showed definite inhibition of diastase and trypsin by oligodynamic action direct from a dilute metal solution and from oligodynamic action transferred to other media. This led Baumgarten and Luger (1917) to believe that the oligodynamic inhibition is caused by a metal salt solution mechanism. Langer (1917) demonstrated that very dilute solutions of silver nitrate and copper sulfate inhibited diastase in living plant cells.

After investigating the effect of ions of silver, mercury and copper on saccharase, von Euler and Svanberg (1921) observed that silver and mercury ions had a greater poisoning effect than had copper sulfate. Since the actions of silver and mercury were quantitatively reversible, they concluded that usually one molecule of saccharase was completely inactivated by 1 silver ion. Egg albumin, cystein and nucleic acids had a strong affinity for silver but it was not as strong as that of saccharase. Händel and Segall (1923) observed that bright copper metal retarded the action of isolated blood catalase, the enzyme that decomposes hydrogen peroxide when added to blood.

Mori (1923) concluded that heavy metals inhibit enzyme activity, not by destroying the enzyme, but by inactivating it temporarily, and that the activity can be restored by removing the inhibitory substances. He found silver effective in greater dilution than copper or gold. Langwell (1932) believed that extracellular enzymes could be inactivated and fermentation inhibited without injury to the organism. The results of a study of the effect of Cu, Hg, Zn, Mn and Fe on proteolytic enzymes indicated to Michaelis and Stern (1932) that these metals can form complexes that deactivate enzymes.

Yudkin (1937) demonstrated that *E. coli* could be killed by silver before there was an appreciable effect on enzymes such as glucose, lactic, succinic or formic dehydrogenases. However, he stated that the opposite had been observed, *i.e.* inhibition of enzyme without impairing growth. Laki (1942) thought that inactivation of fumarase by copper and mercury was the same as denaturization of proteins by heat. Inactivation by iron and silver was different, probably because they are easily reduced and so oxidize the enzyme at S-H groups, a particularly sensitive place.

Vande Velde (1947) studied the effect of neutral metals on urease and found that many had very little effect but that Pb, Cd, Fe, Bi and Zn are clearly toxic and Ni, Mg, Hg, Co, Cu and Ag retard the action greatly.

Ambrose, Kistiakowsky and Kridl (1951) studied the inhibition of urease by  $\text{Ag}^+$  in citrate buffer at pH 5.6 and  $20^\circ\text{C}$ . They thought that inhibition was produced by the reversible reaction of one  $\text{Ag}^+$  with an active site in the enzyme molecule.

*Hemolysis.*—Hausmann and Kerl (1920) observed the hemolytic activity of metal plates embedded in agar plates containing human erythrocytes and found that the activities of Ag, Cu and Mg were especially rapid. Hess and Reitler (1920) found that Cu, Cd and Pb were hemolytic in 5 per cent erythrocyte solution but that Ag, Sn and others had no effect. A 1.0 per cent solution was more



susceptible, and they observed hemolysis in the following descending order: Cu, Cd, Pb, Ag, Ni, Mo, Sb and Al. Sodium chloride solution was made hemolytic by contact with Cu.

In a previous paper Meneghetti (1922) had demonstrated that many metallic salts were fundamentally cationic, the activity varying with their concentration and that high concentrations produced hemolysis and low ones coagulation. With silver and mercury salts hemolysis depended on concentration of metal ions. Compounds that ionized strongly were most active and those that dissociated to complex metal ions were least active. Since hemolysis appeared after removal of the agent from a coagulated mass of blood cells, he thought that the metal was still present in ionic form in fixed erythrocytes and assumed that coagulation was caused by the formation of a combination with metal ions or of an ionic adsorption product.

Of all metals (Au, Ag, Cu, Al, Sn and Fe) tested by Rosenberg (1924) for hemolytic action on erythrocytes in physiological salt solution only copper was active and she was unable to transfer this activity to glass. However, Ball (1933) demonstrated that hemolysis was caused by silver present in the cp sodium chloride used for physiological salt solutions.

*Effect as Catalysts.*—Messerschmidt (1916) thought that metallic impurities in trace metals gave them disinfectant power and acted as catalysts for the solution of the principal metal. Eisenberg (1919) observed that one effect of poisoning was the catalytic destruction of bacterial protoplasm.

The reaction with indicators, the coagulation of albumin, etc. indicated to Falta and Richter-Quittner (1921) that greatly diluted metals can produce various chemical reactions without entering directly into the reaction. Herzberg (1923) believed that bactericidal metals acted only as catalysts for oxidations and dehydrogenations, and Degkwitz (1929) believed that the oligodynamic effect was caused by chemical solution or the catalysis of metal.

*Effect of Altering the Nuclear Oscillation Frequency.*—Lakovsky (1929) believed in the existence of intracellular electrical oscillation of very high frequency, which can be altered by contact with a mass of metal. Therefore, when he found that metal spirals reduced the bacterial count of *E. coli* suspensions to zero in 24 hours he concluded that bactericidal action by metals is purely physical and is caused by the altering of nuclear oscillation frequency.

*Relation of Activity to the Periodic System.*—Messerschmidt (1916) and Neisser and Eichbaum (1932) concluded that the oligodynamic activity of metals is independent of their position in the periodic system. However, Murto (1930) and Horelli (1930-31)

insisted that all members of any one group had approximately equal oligodynamic characteristics, and Murto thought that he could forecast oligodynamic activity of any metal without testing it, merely by comparison with the known action of some metal in the same group.

*Relation of Activity to Gram Specificity.*—Neither Bechhold (1918) nor Eisenberg (1919) could establish a correlation between oligodynamic activity and Gram specificity but Bail (1919) thought that Gram negative organisms were more resistant than were Gram positive ones. Romans (1950) and Ramsey (1951) found Gram positive organisms susceptible to O-Silver but they were slightly more resistant than were Gram negative organisms.

*Relation of Activity to Valence.*—Galeotti (1903) concluded that no combinations between heavy metals and albumin occur that really depend on valence theory, and Tauchert (1931) was unable to establish any regular relationship between the valence of cation and the resulting oligodynamic activity.

*Transport of Metals by the Reticulo-endothelial System.*—Hill and Pillsbury (1939) observed that blood is the chief vehicle for the transport of silver in the body, the major portion being carried in the plasma, probably as silver chloride or silver albuminates. Silver has been found in phagocytes but never in erythrocytes. After administration, the concentration of silver in the blood is gradually reduced until, after varying periods of time, only insignificant amounts remain. The lymphatics are also capable of transporting silver. There seems to be a specific affinity of silver for connective tissue framework and the vascular bed. They thought that silver particles may be deposited either extra- or intra-cellularly and that initial deposition occurs within the cells of the reticulo-endothelial system with extracellular deposition occurring later. It seemed to them that silver is usually taken up within the phagocytes and cells of the reticulo-endothelial system and is then given up after a time, causing it to be found extracellularly.

As a result of his studies of the use of metallic "thermions" introduced into the lungs by insufflation for the treatment of infections and neoplasm, de Kérangal (1947) concluded that the "thermions" were absorbed by macrophages and transported to the site of the infection or neoplasm.

After intramuscular injection of a dextrin protected radioactive silver colloid, Gammil, Wheeler, Carothers and Hahn (1950) found the highest concentration of silver at the site of an infection. Intravenous injection of this colloid into rats and intraperitoneal injection of a gelatin silver colloid into mice caused the highest concentration of silver to be deposited in the reticulo-endothelial

system. Much of the silver was eliminated through the intestines and feces.

West, Elliott, Johnson and Johnson (1950) demonstrated that radio-active silver, from silver nitrate, was localized at a predetermined site in animal tissue. Their experiments showed that the active metal concentrates in areas of spontaneous or induced infection. They thought it possible that the silver was transported by the leukocytes and concentrated in the infected areas as a result of phagocytosis.

*Healing Index of O-Silver.*—Barnes (1952) determined the healing index (per cent wound potential lost per hour) of O-Silver gauze used as a wet dressing by the method described by Barnes, Karasic and Amoroso (1951). It was 2.83 per cent for silver and 2.77 per cent for the controls run at the same time and on the same hand. This indicated that there is no significant difference between treated and untreated wounds (total 16 lesions). Therefore, he concluded that O-Silver is not irritating and does not delay healing. Comparable tests using iodine, sulfanilamide or mercury indicate that they are irritating and can delay healing. On the intact skin of fingers O-Silver-Aqueous applied by dipping the fingers in the solution produced an average positive potential of 3 mv. He thought this was probably due to the positively charged silver adsorbed on the protein of the intact skin (44 measurements on 8 finger tips).

*Pharmacology of Small Amounts of Silver.*—Silver eating and drinking utensils have been used for centuries. Silver and silver compounds have been used for the treatment of drinking water and foodstuffs with no evidence of undesirable consequences, and silver amalgams have been used as restorative material in dentistry without untoward effect. However, excessive amounts of various silver compounds can cause argyria, a discoloration of the skin or tissues. The minimum amount required to do this is much greater than that needed for oligodynamic action, and no report of argyria caused by silver used in oligodynamic amounts has been found in the literature.

After examining 195 specimens of different healthy and diseased human tissues, Sheldon and Ramage (1931) reported that the maximum amount of silver found was .001 per cent of the dry weight. Milla (1937) investigated the physiological action of highly ozonadynized water (2 ppm silver). Daily consumption of rats was 0 gamma silver per 100 grams body weight, equivalent to 37 mg/100 kg for man and much more water than a man can introduce into his system in any manner. Rats remained healthy and lively, all functions were satisfactory and autopsies revealed no abnormality of the internal organs and no granules in the kidneys.



A thorough review of the literature on argyria, led Hill and Pillsbury (1939) to the conclusion that there seems to be no question that silver is a normal constituent of the tissues of some lower animals and man. They thought that all persons tend to accumulate small increments of silver from the use of silver-containing utensils and teeth fillings.

TABLE 63. — PENETRATION OF SILVER INTO SKIN (STOUDT, 1952).

<i>O-Silver treated skin</i>				<i>Untreated skin</i>	
<i>Patient A</i>		<i>Patient B</i>		<i>No silver</i>	
<i>Skin layer</i>	<i>Ag ppm</i>	<i>Skin layer</i>	<i>Ag ppm</i>	<i>Skin layer</i>	<i>Ag ppm</i>
Surface to .002"	5.2	Surface to .008"	3.2	Surface to .005"	0.2
.002" to .014"	4.0	.008" to .018"	1.6	.005" to .010"	0.2
Surface to .022"	0.6	.018" to .028"	1.1	.010" to .015"	0.2
.022" to .028"	0.3	.028" to .038"	0.7	.015" to .020"	<0.2

Kehoe, Cholak and Story (1940 *a, b*) analyzed the food and feces of a normal adult American and concluded that the daily intake of silver was about .06 to .08 mg. They thought that most of it was derived from food containers, cooking utensils and tableware made of silver. Ten per cent of the human tissues analyzed by them contained a small amount of silver, less than .01 ppm.

Ramsey (1950) applied 25 grams of O-Silver ointment per kilogram of weight, each day for 20 days, to the shaved backs of 16 rabbits—5 with burned, 6 abraded and 5 intact skin. The wounds healed, there was no discoloration of skin, the animals gained weight and all functions were normal.

Hodge (1952) found aqueous solutions of O-Silver effective for preoperative skin preparation before mastectomy, and Stoudt (1952) determined the penetration of silver into the skin of these patients by spectrographic analysis of various layers cut with a dermatome (Table 63). The results indicate that the silver in normal skin is more or less evenly distributed in depth while the applied silver shows little penetration; the highest concentration being near the surface of the skin.

Ramsey (1952) tested O-Silver treated paper pulp prepared for use in disposable diapers, using the shaved backs of rabbits, in some instances with abraded skin. The backs of 5 rabbits were exposed continuously to urine saturated, silver treated pulp that was changed twice daily and three controls were treated in the same manner using untreated pulp. Spectrographic analyses of the

ital organs indicated great variation in the silver content but there was no evidence of increased silver in those animals exposed to treated pulp over those exposed to untreated pulp (Table 64). Subsequent tests, Stoudt (1952), showed that rabbit food (Purina Lab Pellets) (R) contained 4.0 ppm silver.

TABLE 64.—CONCENTRATION OF SILVER IN VARIOUS RABBIT TISSUE (RAMSEY, 1952).

Rabbit No.	Treatment skin	Silver (ppm)			
		Skin	Kidney & liver	Lymph nodes & spleen	Tissue & bones
1	Ag-abraded	6.2	0.12		
5	Ag-abraded	0.5	2.7	6.9	0.02
2	Ag	0.053	0.01		0.01
6	Ag	0.9	2.8	14.5	
7	Ag	2.1	6.3	48.9	
8	untreated	3.0	1.3	50.8	
9	untreated	2.5	1.0	4.1	
0	untreated	1.6	0.6	20.7	

## OLIGODYNAMIC PROCESSES AND PRODUCTS

*Katadyn Silver.*—The oligodynamic activity of metals has been the basis for the development of many antimicrobial processes and products. One of the earliest was "Katadyn Silver" developed by Krause (1928). Rochat and Uzdins (1947) suggested that the name was derived from the German and means "silver preparation that has catalytic oligodynamic effect." Krause described it as a spongy, mellar, metallic form of pure silver to which is added an activating metal below silver in electropotential series, such as palladium or gold. It can be used for linings of flasks, "Katadyn Flasks," as impregnation on filter elements or as coatings on sand or other materials. It was developed to provide an optimum ratio of silver surface to silver volume and thus supply maximum antibacterial and catalytic activity. After several weeks contact with silver, water contained 15 gamma/L and was oligodynamically active even after dilution to one tenth of the original concentration. Krause considered this to be the most active form of silver, and he found inhibition by low concentrations of various salts, or 0.2 per cent quillon, or 0.01 per cent serum, or 0.01 per cent glucose, and sterilization was independent of temperature.

Konrich (1929) found that the antibacterial activity of Katadyn Silver covered sand was faster than that of other oligodynamic bactericides. It could handle much larger concentrations of bacteria than they could. In addition, temperatures between 20° and 37°C had no effect, but lower temperatures were inhibitory. Schweizer

(1929) verified Krause's findings that Katadyn can kill pathogens and *E. coli* but not harmless air and water bacteria. He thought that bacteria vary in sensitivity at different times. Suckling (1931) observed that Katadyn was more efficient than chlorine for disinfection of aerobic sporulating bacilli, *Bacillus mycoides* and *Bacillus subtilis*.

Bigger and Griffiths (1933) found that from 15 grams silver sand per liter water adsorbed 6 gamma of silver and could kill

TABLE 65. — EFFECT OF KATADYN FLASKS ON WATER CONTAINING VARIOUS NUMBERS OF *E. COLI* (SÜPFLE AND WERNER, 1951)

Water treatment	<i>E. coli</i> per ml	Bacterial count after standing at room temperature						
		1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	24 hours
In	1,500	90	30	0	0	0	0	0
"Katadyn	18,000	3,800	1,500	150	0	0	0	0
Flask"	120,000	51,000	17,400	20,000	9,100	2,600	1,100	0
Control	18,000	18,000	18,000	18,000	18,000	18,000	18,000	12,000

TABLE 66. — EFFECT OF STORAGE ON THE ANTIBACTERIAL ACTIVITY OF KATADYN FLASK (SÜPFLE AND WERNER, 1951)

Length of storage	<i>E. coli</i> per ml	Bacterial count after standing at room temperature						
		1 hour	2 hours	3 hours	4 hours	6 hours	7 hours	8 hours
2 hrs	18,600	1,080	120	60	0	—	—	—
12 "	22,500	120	90	30	0	0	—	—
24 "	17,200	420	90	0	0	—	—	—
2 days	10,400	180	0	0	0	—	—	—
8 "	7,700	360	0	0	0	—	—	—
Control	29,400	—	—	—	—	24,000	—	—
	10,800	—	—	—	—	—	—	9,000

*E. coli* in from 2 to 24 hours, depending on the number of bacteria. After investigating the action of silver-covered porcelain rings Gibbard (1933a) concluded that oligodynamic activity is inhibited by high concentrations of bacteria and low temperatures. Further study (1933b) showed that the activity of these rings was inhibited by the presence of organic matter and inorganic substances, so consequently, he could not recommend them for the treatment of water.

Moiseev (1934) concluded that, in spite of fouling, silver sand acted vigorously for a long time, could sterilize water containing large numbers of bacteria, could kill pathogens more easily than typical saprophytes of air and water, and that after fouling, activity could be restored by mechanical cleaning. He thought that the weight of water being treated and the weight of silver on the sand was much less important than the amount of active silver surface exposed to the water.



While testing silver-impregnated filter elements, Herrmann (1934) was unable to get more than 93 per cent elimination of poreforming bacteria, yeasts and molds. Piazza (1934) concluded that "Katadyn Flasks" were not practical for military use because the bactericidal effect varied with contact time, the genus of bacteria and the amount of silver surface. It was inhibited by milk; it had no effect on protozoa; and there was no good bactericidal indicator available.

TABLE 67.—INFLUENCE OF TEMPERATURE ON THE BACTERICIDAL EFFECT OF SILVER ACTIVATED WATER (SÜPFLE AND WERNER, 1951).

Temp. ° C.	Silver gamma/l	<i>E. coli</i> per ml	Bacterial count after standing			
			1 hour	4 hours	6 hours	24 hours
22	500	14,000	240	0	0	—
	100	12,300	1,260	0	0	—
	10	10,800	3,000	60	0	—
	0	24,600	25,000	15,600	16,700	—
0	500	26,000	16,900	2,700	780	0
	100	19,000	15,000	2,500	1,500	0
	10	27,000	18,000	4,600	3,800	120
	0	22,000	26,000	21,000	27,000	22,000

Süpfle and Werner (1951) observed that spores remained viable for more than 5 weeks in a mixture of 200 grams Katadyn quartz sand (10 per cent Ag), 270 ml of tap water and 30 ml of suspension of *Bacillus mesentericus* spores. Therefore, one should not speak of "sterilizing" water or other fluids with the Katadyn process. However, they did not think that this should preclude the possibility of considering the silver treatment of drinking water because the only infection by pathogenic spores carried in water was in the case of anthrax and then it was only in animals.

They tested the effect of increasing numbers of *E. coli* (survival in 1 per cent phenol for 90 minutes) in samples of water stored in "Katadyn Flasks" and found that it delayed antibacterial activity (Table 65). They demonstrated that increased storage time up to 24 hours increased the bactericidal effect, and that there was no change after that (Table 66). Prolonged contact with Katadyn porcelain rings, marbles or sand did not cause a similar increase in activity. After 2 days contact the water contained 500 gamma silver per L and could kill 8,000 *E. coli* per ml in 2 hours. There was no increase in silver concentration and no decrease in killing time after 1 month's contact.

They demonstrated the inhibiting effect of low temperatures at various silver concentrations (Table 67). They showed that a silver activated solution becomes less bactericidal as the silver is adsorbed on glass by inoculating at intervals three glass containers containing silver activated water (500 gamma silver per L) with 500,000 *E. coli* per ml. The first was inoculated immediately, the second after 24 hours and the third after 48 hours. The first killed *E. coli* in 3 hours, the others required longer contact (Table 68).

TABLE 68.—INHIBITION OF THE BACTERICIDAL ACTION BY ADSORPTION OF SILVER ON THE GLASS WALL OF A CONTAINER

Length of contact with glass	<i>E. coli</i> per ml	Bacterial count after standing at room temperature				
		2 hours	3 hours	4 hours	5 hours	24 hours
0	500,000	120	0	0	0	0
24 hours	500,000	240	90	30	0	0
48 hours	500,000	30,000	24,000	20,000	10,000	0

TABLE 69.—EXHAUSTION OF THE BACTERICIDAL ACTION OF SILVER ACTIVATED WATER (500 GAMMA/L) BY REPEATED INOCULATION—ROOM TEMPERATURE (SÜPFLE AND WERNER, 1951).

	<i>E. coli</i> per ml	
	50,000	500,000
First Inoculation	50,000	500,000
After 24 Hours	0	0
After Centrifuging, Second Inoculation	50,000	500,000
After 2 Days	0	0
After Centrifuging, Third Inoculation	50,000	500,000
After 3 Days	6,000	30,000
After Centrifuging, Fourth Inoculation	50,000	500,000
After 4 Days	30,000	40,000

Similar results were obtained when silver was adsorbed by large numbers of microorganisms. This was illustrated by repeated inoculation of *E. coli* in water strongly activated by silver, 500 gamma silver per L. After 1, 2, 3 and 4 days the mixtures were centrifuged, the supernatant was poured into another glass and inoculated again (Table 69). This is reported as the effect of microorganisms only but, since the glass container was changed every 24 hours, it is probably the combined effect of microorganisms and glass.

They demonstrated the inhibiting effect of inorganic substances and colloids by increasing the hardness of water and by adding 1 per cent bouillon to it. Five hundred gamma silver per L was sufficient to kill 8,000 *E. coli* per ml added to Dresden tap water in 2 hours. This was not accomplished in 3 hours when interfering materials were present (Table 70).

The inhibiting effect of small amounts of impurities in Dresden tap water was shown by a comparison of the effect of various concentrations of silver in distilled water and Dresden tap water. While tap water containing 500 gamma silver per L did not kill 23,000 *E. coli* per ml in 6 hours, distilled water with same silver content did so in 1 hour (Table 71).

TABLE 70.—INHIBITING EFFECT OF INORGANIC SUBSTANCES AND COLLOIDS ON THE BACTERICIDAL ACTION OF SILVER ACTIVATED WATER (550 GAMMA/L) (SÜPFLE AND WERNER, 1951).

Composition of water		<i>E. coli</i> per ml	Bacterial count after standing at room temperature		
			1 hour	2 hours	3 hours
Dresden Tap Water	Silver	8,200	90	0	0
8.6° d.h.	Control	9,800	12,600	12,000	9,600
Hardened Water	Silver	6,500	2,400	360	30
25° d.h.	Control	8,400	6,900	8,900	8,700
Water Containing	Silver	10,500	960	120	30
Colloids	Control	8,200	9,000	9,900	9,300
1% Bouillon					

TABLE 71.—COMPARISON OF THE BACTERICIDAL ACTION OF DISTILLED WATER AND TAP WATER WITH THE SAME SILVER CONTENT (SÜPFLE AND WERNER, 1951).

Type of water			Bacterial count after standing at room temperature			
			1 hour	4 hours	6 hours	24 hours
Distilled	500	13,000	0	0	0	—
	100	20,000	1,590	0	0	—
	10	19,000	8,100	850	680	—
	0	23,000	16,000	14,500	15,100	—
Dresden Tap Water	500	26,000	16,900	2,700	780	0
	100	19,000	15,000	2,500	1,500	0
	10	27,000	18,000	4,600	3,800	120
	0	22,000	26,000	21,000	27,000	22,000

For the treatment of liquids, Katadyn Silver can be held in a suitable glass chamber and the liquid to be treated run through it at a rate that will produce the desired effect. Brandes (1934) described such an apparatus and reported that a concentration of silver as low as 0.015 mg/L had a definite effect on the acid-ester ratio of alcoholic liquids, improving the odor and taste of raw alcohol, thus making the process useful to both the perfumery and brewery trades.

Silver ions can prevent the clouding that results from continuous growth of microorganisms in liquids produced by fermentation and they have been used successfully for the treatment of vinegar,



cider and wine, Kreipl (1934), Gretler (1934) and Perin (1948 a, b).

Clinical studies using Katadyn powder (particles  $2\ \mu$  by  $1/100\ \mu$  containing 10 per cent silver) were made by Rochat and Uzdins (1947). The results obtained by others using this product in a paste to treat root canals, in a spray to treat tonsilitis and as a powder or in water suspension to treat abrasions and burns caused them to think it might be useful in seriously infected lesions. After using Katadyn powder combined with  $O_2$  or  $CO_2$  for the treatment of severe burns, carbuncles and infected wounds, they concluded that Katadyn succeeds where other treatments fail. It promotes tissue granulation, suppresses fetid odors of wounds, accelerates healing and prevents keloid formation.

*Electro-Katadyn Process.*—Small volumes of water or other liquids can be treated by the so-called "pocket-size" Electro-Katadyn. One such unit described by Brandes (1934) consists of 2 plates of silver mounted close together on a synthetic plastic handle containing a tiny dry cell. Sufficient silver ions can be produced in 60 to 80 seconds to sterilize 1 L of water within 1 hour. Karsten (1935) described a similar unit that could sterilize 1 L of water per minute and reported that a 3 minute contact imparted residual sterilizing power. Using suspensions of several different bacteria as test solution, Schioppa (1936) demonstrated that 260 gamma silver per L was optimum dose and it required 1 hour for sterilization of 50,000 bacteria per ml and longer intervals for larger number of bacteria. Chuda (1937) studied the effect of silver and copper electrodes in this unit and concluded that silver was more active than was copper for all bacteria tested. The silver dose averaged about 0.175 ppm.

For larger quantities of water, a chamber containing a battery of silver plates with a clearance of 15 mm is placed in line containing flowing water and a direct current is induced across them (Brandes, 1934). This current is reversed frequently to prevent the formation of anode coatings of high resistance. The current is used to increase the solution pressure of metallic silver and thus shorten materially the time necessary to reach the desired concentration of silver. This concentration is determined by the size of the chamber, number and area of plates, the rate of flow, the conductivity and pH of the water and the current passed (1 mg silver dissolved per 0.5 milliampere-hour). After the treatment, sterilization is accomplished by storing the water for appropriate periods. The concentration of silver ions required depends on the condition and use of the water. Clear drinking water, pH 7.0, requires 0.05 mg per L to kill all bacteria within 1 hour. Turbid water requires a higher

dose, or longer contact. Swimming pool water can be kept permanently sterile by a concentration of 0.15 mg per L in the make up water added daily. An effective, non-corrosive, harmless germicide, which can be used for sterilization by washing may range from 0.100 to as high as 0.900 mg per L. This type of solution is used for washing hose, filters, bottles and other utensils in the brewing and dairy industries. Water treated before freezing becomes sterile and able to disinfect fish or water cooled with the ice. Brandes concluded that the initial cost for water treatment is greater than that for chlorine but advantages such as the absence of added taste and odor practically balance this and it is cheaper than sterilization by ultra-violet light.

Viesohn (1933) observed that after the pool walls and filter had absorbed some silver thorough bactericidal and algacidal action was obtained from Electro-Katadyn. An initial dose of 200 to 400 gamma silver per L was required for sterilization.

Gutschmidt (1934) observed that there is an optimum current to produce a dose of silver above which no improvement in bactericidal action was observed. After a review of the work of previous investigators, Rogers, Schoonover and Jordan (1936) concluded that Electro-Katadyn was outstanding and observed that silver is bactericidal in one half to several hours. Bertarelli, Caserio and Peragallo (1936) found that aeration of water prior to treatment with Electro-Katadyn prevented subsequent inhibition of the bactericidal action by hydrogen sulfide. They observed that the use of a stronger current than necessary produced change in the color of the water. Hoffmann (1938) working with Zurich tap water and about 2 million *E. coli* per ml found that sterility was produced by 0.25 ppm silver after 3 hours contact. Disinfection was equally rapid for like amounts of silver ions from silver nitrate.

Most of the reports on use of Electro-Katadyn are about its use for the treatment of swimming pools. Two of the most outstanding and well-documented ones being those of the Congressional Country Club, Washington, D. C., Anonymous (1934) and Doroh (1935), and the Hotel Palazzo della Fonte, Fiuggi, Italy, Alessandrini and Labranca (1939) and Frongia and Napoli (1941). Both installations operated successfully, and it was concluded that even though complete sterilization was not obtained it was an improvement over other methods because of its long time residual effect and non-production of odors and tastes.

After a study of Katadyn treated swimming pool water which revealed that *E. coli* tests were preponderantly negative but the bacterial counts were not affected and contained *M. pyogenes* var. *aureus* and *albus* and streptococci which may be important in eye,

ear, nose and throat infections, Shapiro and Hale (1937) concluded that such treatment was impractical for swimming pools.

Judging on basis of A.P.H.A. standards, Mallmann (1937) found that all water samples from Electro-Katadyn treated swimming pools showed unsatisfactory bacterial counts (increased from 0 to 205,000 per ml) but *E. coli* indices passed (remained negative). He found that silver resistant bacteria were not disease producers, had no relation to pollution by bathers and were common air and water bacteria, but he concluded that silver did not provide a satisfactory treatment for swimming pools.

Goetz *et al.* (1940) observed that if silver ions are brought into solution by an electrolytic current, silver concentrations can be obtained which are far above the solubilities of any known silver compound possible under the circumstances. Since such solutions are unstable, they assumed that the metal is not fully dissociated but exists in a state of molecular dispersion; as a hydrosol which is only partly ionized.

Hoffmann (1947) reported that the treatment of swimming pool water with silver involves first, an "impregnation" with several hundred gamma per L followed by an "activation" by at least 50 gamma per L. The *E. coli* and total counts were considerably reduced by this treatment, which he thought both economical and effective.

*Matzka Process.*—This method for the introduction of silver ions into liquids such as fruit juices and wines combines temperature differential and electro-potential differential produced by contact of a medium with two different metal plates, a silver-gold alloy as the oligodynamic anode and nickel or aluminum as the neutral cathode. Goetz (1940) thought that this method was successful but that the apparatus was complicated and its function not very apparent.

*Cuma System and Silbermolke.*—The name "Cuma" was coined by Kruse and Fischer (1936) (1937) from "cum argentum"—(with silver) to indicate that their method is a combination of silver and chlorine. It is an electrolytic method producing chlorine in conjunction with electrolytic production of silver ions. Most waters contain sufficient chlorides for the process but if not, 0.05 ppm sodium chloride may be added. The apparatus is manufactured in several sizes, small or pocket size, larger for household use and still larger for installation in swimming pool or drinking water systems. After installation in a swimming pool *E. coli* was never found in 100 ml. However, silver resistant saprophytes and low bacterial counts were observed. Kruse (1939) contended that these bacteria were harmless and their counts had no relation to bather population.



He thought that evaluation of a disinfectant should be on the basis of the *E. coli* index. Bacteria counts are unreliable since they fluctuate from day to day, while *E. coli* remain continuously absent. Therefore, bacteria counts can have no importance from hygienic standpoint. He observed that 10 gamma silver per L was definitely algaecidal.

Kruse and Fischer (1934 *a, b*) used a modification of this system for activation of silver plated instruments by electrolysis in chloride or carbonate medium, thereby forming active silver chlorides or silver carbonates on silver surfaces. They also developed a wound sterilizing liquid "Silbermolke" (milk of silver) containing electrolytically produced silver chloride and a protective colloid. Oertel (1934) tested the effect of this liquid on *E. coli* and staphylococci in bouillon. The results indicated stimulated growth at low silver concentrations. With further dilution, growth varied between retardation and stimulation. Ten ppm Silkermolke killed *E. coli* after 10 hours contact.

Oosterholt and Rodenburg (1939) reported effective removal of slime caused by iron bacteria and fungi that collected at dead-ends, clogged Venturi and Waltman meters and floated on the surface of clear wells by the use of the Cuma System with a dose of 30 grams silver per 264,000 gallons of water. Suspension of treatment for the application of copper sulfate resulted in slime which disappeared 2 weeks after resuming the silver treatment.

*Uglov Black Silver Sand*.—A sand coated with silver-manganese was developed by Uglov and Gan (1935). They believed that the silver-manganese compound was slightly more soluble than silver metal and, therefore, more active. The coating contained 1.37 per cent silver and 0.037 per cent manganese. They calculated that 10 kilograms of "black" sand should sterilize 38 tons of water. Uglov and Uglova-Ovchinnikova (1935) prepared a filter column containing this material. Tests using infected river water showed a reduction of *E. coli* from 9,500 per ml to 0 within 6 hours from the start of filtration. Granata (1946) treated charcoal for filter elements by this method and reported greater efficiency than that obtained from silver sand.

*Heavy Metal Aerosols*.—Expecting a greater bactericidal action from aerosols of metal compounds on account of the colloidal dispersion of the solid phase and probably also on account of their increased solubility, Kliewe *et al.* (1947) prepared aerosols, the disperse phases of which were solids and the dispersing agents gases. They found silver chloride the most effective compound for this purpose and that silver chloride aerosol produced with air kills *S. typhosa* on agar within 1 to 2 minutes, *E. coli* 2 to 3 minutes,

staphylococci within 10 minutes and anthrax spores within 15 to 30 minutes. Bactericidal action was not decreased by the presence of serum, blood, saliva or meat. They found that this aerosol could be used for the preservation of agglutinating sera, complement or amboceptor without affecting their activity.

*Aero-thermo-metallo-iono-therapy.*—de Kérangal (1939) reported the successful treatment of oral, jugal and lingual leukoplakia with aero-thermo-metallo-iono-therapy using copper “thermions” produced in what he called an “Athmik apparatus” which he did not describe. Later (1946) he reported the successful treatment of the chronic catarrh of mucous membranes of the nasal cavity, chronic otorrhea and cervical and uterine metritis by insufflation of “thermions” of silver and of asthma and spasmodic head colds involving hydrorrhea by insufflation and inhalation of silver “thermions.” He suggested that the latter might be used in the preventive treatment of diseases, such as poliomyelitis, in which infection is produced through nasal mucus. In another article (1947) he reported successful treatment of malignant neoplasms and benign neoplasms susceptible to becoming malignant, of the throat, intestines and breast by insufflation and inhalation of copper “thermions” into the lungs. He explained that this action resulted from absorption in the lungs, transportation by circulatory system, fixation in connective and reticulo-endothelial system surrounding the tumor and finally penetration into its interior.

*Fissan-Silver Powder.*—Scheider (1951) reported clinical studies on the use of Fissan Silver Powder for the treatment of wound and skin diseases. It contains silver linked to an unstable milk albumin and mixed with a Fissan colloid to extend surface area. Satisfactory results were obtained from its use for the treatment of fresh cuts, wounds, leg ulcers, pemphigus, herpes zoster, decubitus and decubital ulcers, furunculosis, etc. This convinced him that it is strongly bactericidal, astringent, secretion absorbing, detoxicating and a stimulant to recovery. He concluded that it is as effective as sulfonamide or penicillin powder as to bactericidal action and rapidity of recovery and much cheaper than either of them.

*Movidyn.*—Movidyn was evaluated by its bactericidal activity toward different microorganisms by Hoffman, Surkiewicz, Chambers and Phillips (1952). Comparative tests using *E. coli* demonstrated that the silver was in a more active form than it was in silver nitrate. The use of 0.1 per cent sodium thioglycollate in the plating medium indicated that the action was bactericidal; 0.1 ppm killed *Serratia marcescens* in 1 hour, *S. typhosa* in 3 hours, *M. pyogenes* var. *aureus* in 5 hours and *E. coli* in 7.5 hours. Chambers

Chambers and Kabler (1952) determined the effects of some environmental variants on the bactericidal action of Movidyn. They reported that under relatively favorable conditions of pH 8.5 to 9.0 and temperature range 22° to 25°C, 99.9 per cent of the test organisms may be killed by as little as 0.2 ppm Movidyn in 4 hours. When other conditions are constant, the killing power is much greater at pH 8.5 to 9.0 than at pH 6.0 to 6.5. Lowering of temperature from the 22° to 25°C range to the 2° to 5°C range appeared to reduce the efficiency more than has been observed with some other disinfectants. The bactericidal action of Movidyn in two natural waters and one synthetic water exhibited appreciable variation. The constituents of water that caused the variations were not identified.

*O-Silver*.—O-Silver, Romans (1949), is an aqueous solution containing oligodynamic silver that can be adsorbed on a variety of materials including hair, skin, mucous membranes, glass and cellulosic materials, such as paper, cotton, wood, cellophane, etc. It can be used as an aqueous or alcoholic solution or incorporated in a lotion or ointment base. O-Silver treated paper fibers used in disposable diapers were shown to prevent and cure infantile diaper rash, Torres, Wheeler and Romans (1951). O-Silver Ointment was found effective in the treatment of burns and infected wounds, Hodge (1951). O-Silver, Aqueous was found effective in preoperative skin preparation of the patient, Hodge (1952) and caused significant reduction in the numbers of bacteria on the hands of persons using it for wiping tables and the like, Ramsey (1952). After removal during mastectomy skin treated with this solution showed the following zones of inhibition on agar plate: *M. pyogenes* var. *aureus*, 5 mm; *Pseudomonas aeruginosa*, 4 mm; *E. coli*, 4 mm; *Streptococcus pyogenes*, 4 mm, Romans (1951).

Breeding mice, suffering from diarrhea so severe that the exudate extended half way up the back causing loss of hair, were sprayed twice a week with O-Silver aqueous solutions, Romans (1950). During the period of the test (3 months) 122 healthy mice were weaned from the 130 mice on test and new white hair replaced that which had been destroyed because of diarrhea (Fig. 14 (1-7)). Ten days after the last treatment one of the mice was immersed in M-Q developer (Fig. 14 (8)). The body of the mouse turned black but the face remained white. Indicating that while silver had been adsorbed over the entire body, the mouse had been able to remove that on the head by normal "washing" procedures. Chemical analysis of the skin of a mouse receiving similar treatment, but no developer, showed 1.5 mg silver per 1.4 grams of skin. Agar plates, using approximately 10x10 mm pieces of such skin,



showed the following zones of inhibition: *M. pyogenes* var. *aureus*, 4.0 mm; *E. coli*, 5.0 mm and *Ps. aeruginosa*, 5.0 mm. (Page 417).

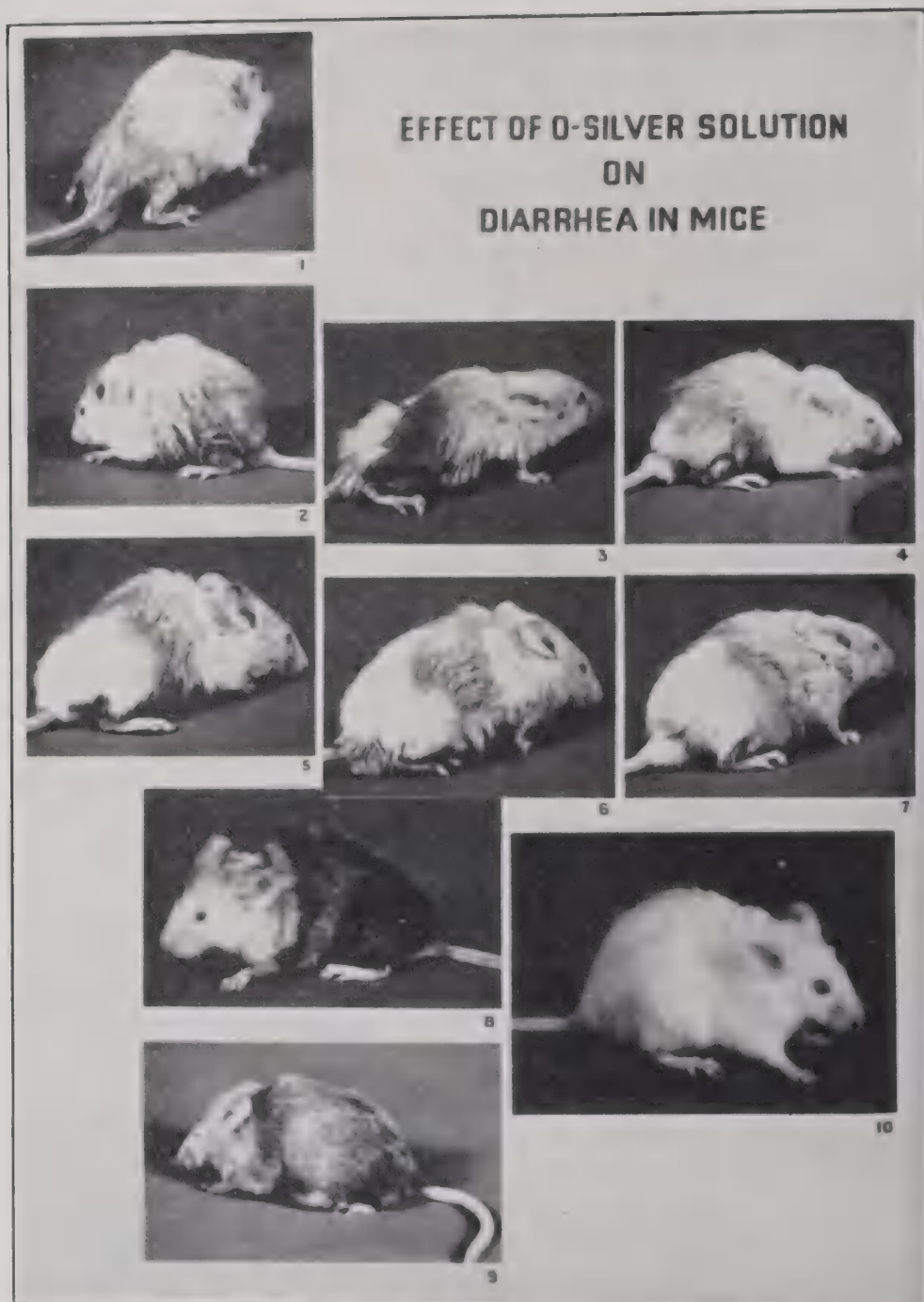


FIG. 14.

Other mice were sprayed once with the same solution. After they were completely dried, one was immersed in M-Q developer. All the hair of the mouse turned dark including that on its face and

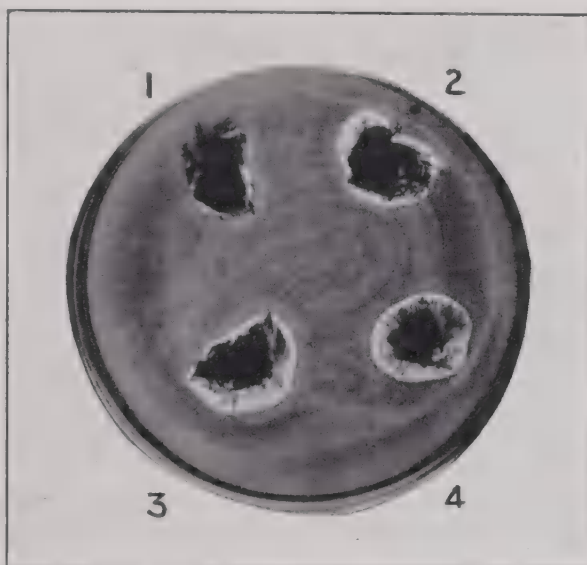
head (Fig. 14 (9)). Chemical analysis of the skin of another mouse treated in the same manner showed 1.8 mg silver per 2.01 grams

1. Triton X-100 solution used once.

2. Triton X-100 solution used 3 months.

3. Polyvinyl acetate solution used 3 months.

4. Methocel solution used 3 months.



*M. pyogenes* var. *aureus*



*Pseudomonas aeruginosa*

*Escherichia coli*

Fig. 15.—Skin from O-Silver treated mice; agar plate test.  
0.5 ml 24 hour broth culture per 100 ml Heart Infusion Agar,  
20 ml agar per plate. Incubation 37° C 48 hours.

of skin. Agar plates made from the skin of a third mouse treated in the same manner gave the following zones of inhibition: *M. pyogenes* var. *aureus*, 2.0 mm; *E. coli* 3.0 mm; *Ps. aeruginosa*,

3.0 mm (Page 417). There was no discoloration of hair due to the O-Silver Solutions. Figure 14 (10) shows a mouse that had been treated twice a week for 3 months. The picture was taken 10 days after last treatment. This mouse had no exudate from diarrhea, so there is no discoloration from that either.

## OTHER PRODUCTS AND PATENTS

It would be impossible to describe all the oligodynamic compositions and processes that have been developed, so only a few representative ones will be described briefly. Hottinger (1922) reduced silver salts in colloidal solution to deposit silver in a spongy state on carriers to which it adheres. This material can be used in a variety of ways, such as on filters, bandages, dialyzing membranes, insoluble powders, etc. Bechhold (1925*b*) impregnated filters with metal compounds, such as silver chloride and activated them by the addition of gold or platinum. Schreier (1927) coated quartz sand and other granular materials with metals, such as silver, copper and gold, by using a solution of the metal and a solution of a reducing agent, such as sugar or formaldehyde or an alkali, such as ammonia. The coated particles are used in filter beds for the sterilization of liquids.

Dieck and Schiff (1935) treated metallic silver with potassium permanganate to impregnate a carrier with silver oxide and manganese oxide. Their products are used for the sterilization of water and the preparation of bactericides for therapeutic use. Feigl (1936) prepared a homogeneous mixture of finely divided metallic silver and manganese dioxide to be used for therapeutic, disinfectant and sterilizing purposes. Later (1940) he increased the efficacy of this mixture by the addition of gold in quantities up to one tenth the amount of silver present. Gallaher (1936) developed "Super-Ionite," a filter material made up of synthetic granular products composed of silver in combination with a base-exchange silicate.

Schiff (1942) prepared antiseptic articles, such as ceramic containers, filters and bandage materials, also antiseptic preparations, such as tooth fillings, powders, ointments, suppositories, etc., containing silver oxide and manganese oxide which he referred to as "silver-manganite." Conconi (1942) prepared oligodynamic filters by saturation with silver nitrate and reduction of the nitrate with formaldehyde gas. Kreidl and Kreidl (1945) impregnated cloth and produced an antiseptic powder by the reduction of a reducible metal compound with a metal formate. Later (1946) they prepared sterilizing materials, such as cloth or gauze by treating the material with a soluble silver compound, such as silver



nitrate, followed by a soluble halide, such as sodium chloride, and after a period of time washing off the excess silver chloride that was not adsorbed on the material.

Burkser (1947) observed that silver activated water could be used for the inactivation of brucella suspensions and that antigens prepared with silverized solutions gave distinct, sharp reactions while those induced by the controls (inactivated by heat) were not as clear cut.

Schwarz (1949a) impregnated organic and nylon fibers with "yellow, highly dispersed, colloidal silver" by reducing, in contact with them, aqueous solutions of a silver compound with alkylolamines. Later (1949b) he produced similar materials by the reduction of the silver solutions with heterocyclic secondary amines, such as piperidine, pyrrolidine, etc. Snell and Shapiro (1949) developed a portable plastic water disinfectant unit containing bactericidal silver coated plastic granules to sterilize water drawn through the unit.

Newton and Jones (1949) observed that silver from silver foil electrodes and solutions of silver nitrate destroyed cysts of *Endamoeba histolytica*, but they felt that both the exposure and the concentrations required were too great for practical drinking water treatment. Barnes and Ham (1948) washed an anion resin with a dilute solution of silver nitrate until the resin contained approximately 8.5 per cent silver. The treated resin was formed into tablets that can be used to sterilize water. Banks and Barnes (1950) extracted silver from a silvered ion exchange resin with formaldehyde and then treated the silver containing formaldehyde with melamine to form a moldable resin. This resin may be extruded into tablets or molded into various vessels, such as tumblers, pitchers, etc. that can remove or reduce the number of bacteria in fluids which come in contact with them.

Mendenhall (1950) prepared glassy phosphates having a metal oxide and phosphorus pentoxide ratio of 1:1 to 2:1 and having a silver content not exceeding 5 per cent, but sufficient to impart germicidal properties when the material is dissolved in water. This composition may be combined with a water soluble soap or a detergent to form an antibacterial cleanser that is effective against both Gram positive and Gram negative organisms in concentrations as low as 0.5 ppm. Goetz (1950) formed antimicrobial complexes by permitting mixtures of a water sludge of finely divided silver oxide and finely divided zinc oxide to digest for a short period of time. The mixture may then be dried and made into granules, pills or pellets. These complexes, in the form of pastes, may be used as germicides for the treatment of burns, wounds or skin

infections. They may be dispersed as a pigment in a plastic binder and used to produce self-sterilizing surfaces on glass, wood, metal, paper, cellophane, etc. Edlinger (1950) observed that "Photonic," a filtrating mass, can sterilize water and that after filtration (contact for 1 to 2 minutes) the water can be used at a dilution of 1 to 100 to disinfect untreated water. The material is composed of silver activated by radiations of low frequency and precipitated on limestone (0.5 to 1 mm granulation) or asbestos.

Lindenmeyer (1951) developed an antiseptic bandage material consisting of very fine threads (diameter 0.03 to 0.08 mm) of metals, such as silver and zinc. The metal threads may be used alone, twined with textile fibers or joined together by cellophane. Fuchs (1951) mixed silver salts and amino alcohols under controlled temperatures to form antibacterial silver complexes that can be used to prepare self-sterilizing fabric, porcelain, bristles, etc. The product can also be incorporated in ointments, salves, etc. for therapeutic use. Quinn (1952) prepared a composition for water purification consisting of a mixture of an inactive carrier, such as carbon granules or sand, coated with silver chloride, activated carbon and an ion exchange resin. The concentration of silver used is sufficient to destroy bacteria developing in the adsorptive materials and resin granules during periods of non-use of the purification unit.

## SUMMARY

The results of some of the extensive investigations into the mechanism and effects of oligodynamic metals, the processes that have been used to produce and enhance this effect, and some of the products containing oligodynamically active metals have been reviewed. These results show that there is an oligodynamic form of metals, particularly of silver, that is antimicrobial and stable. In addition, it is non-toxic to man. It has been successfully used in a variety of ways, such as treatment of water supplies, production of antibacterial and self-sterilizing surfaces and preparation of antibacterial powders, ointments, solutions, etc. for use as therapeutic agents.

However, there is much difference of opinion as to the form of the active principle, as to the mechanism of its action, and the value of the results obtained. Some authorities believe that the active form is a positively charged ion, some think it is a complex ion, others a salt and still others think it acts by formation of proteinate or merely as a catalyst. These are only a few of the ideas that have been expressed with formidable experimental support. So it appears possible that investigators have not always been

working with the same form of the metal and the results are no more comparable than would be expected from investigations of chlorine, chloramines (monochloramine and dichloramine), organic chlorine compounds (azochloramide, chloramine T, succinchlorimide, etc.) and sodium chloride. When these data can be correlated, the usefulness of silver will be more clearly understood.

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## 21

# PHENOLIC COMPOUNDS

### INTRODUCTION

ALTHOUGH phenol (carbolic acid) no longer plays any significant role as an antibacterial agent, its use has not been abandoned entirely. However, more importantly, over a period of many years it has been the subject of extensive studies ever since its adoption by Lister in 1867 as the germicide *par excellence*. The variations in the antibacterial behavior of phenol solutions as produced by changes of concentration, of temperature, of pH, and of other physical and chemical factors are the subjects of numerous papers which have contributed to the elucidation of its mode of action; they have aided also toward the understanding of the action of the numerous phenol derivatives.

For the purpose of this chapter an exhaustive treatment of the very extensive work concerned with the properties of phenol itself in their different applications is deemed to be unnecessary. However one or another characteristic quality of phenol might be underscored at this point by way of placing its mode of action into contrast with that of other types of disinfectants.

One such characteristic is the concentration exponent. Watson (1908) showed on the basis of experimental data supplied by Chick (1908) that the relation of the death rate of bacteria (under the influence of a disinfectant) to its concentration may be expressed by the equation  $c^n t = a$  (const.), where  $t$  is the killing time,  $c$  the concentration and  $n$  the characteristic exponent. The value of  $n$  varies greatly with different disinfectants. Its significance will appear, *e.g.*, from the comparison of phenol with formaldehyde. According to Rahn (1943, 1945), at a concentration of 0.7 per cent both substances kill *Escherichia coli* in about the same time of 100 minutes. However, if the concentration is doubled, formaldehyde with its concentration exponent of 1 will kill in 50 minutes while

phenol with its concentration exponent of 6 (Jordan and Jacobs 1944, 1945) will kill in  $100/2^6$ , i.e., in only 1.6 minutes.

This striking difference between phenol and formaldehyde should not be understood to signify that within the large and varied group of phenol derivatives the concentration exponents are free from variation. In the first place there is no perfect agreement

TABLE 72. — MICROBICIDAL ACTION OF PHENOL DERIVATIVES  
(PHENOL COEFFICIENTS  $37^{\circ}$  C.).

	<i>Salmonella</i> <i>typhosa</i>	<i>Micrococcus</i> <i>pyogenes</i> var. <i>aureus</i>	<i>Mycobacterium</i> <i>tuberculosis</i>	<i>Candida</i> <i>albicans</i>
Phenol (I)	1.0	1.0	1.0	1.0
2-Methyl I	2.3	2.3	2.0	2.0
3-Methyl I	2.3	2.3	2.0	2.0
4-Methyl I	2.3	2.3	2.0	2.0
4-Ethyl I	6.3	6.3	6.7	7.8
2,4-Dimethyl I	5.0	4.4	4.0	5.0
2,5-Dimethyl I	5.0	4.4	4.0	4.0
3,4-Dimethyl I	5.0	3.8	4.0	4.0
2,6-Dimethyl I	3.8	4.4	4.0	3.5
4- <i>n</i> -Propyl I	18.3	16.3	17.8	17.8
4- <i>n</i> -Butyl I	46.7	43.7	44.4	44.4
4- <i>n</i> -Amyl I	53.3	125.0	133.0	156.0
4- <i>tert</i> -Amyl I	30.0	93.8	111.1	100.0
4- <i>n</i> -Hexyl I	33.3	313.0	389.0	333.0
4- <i>n</i> -Heptyl I	(16.7)	625.0	667.0	556.0

Figure in parenthesis approximate

among the investigators reporting these data for any particular compound. However, beyond this there exist substantial differences between individual phenolic compounds as illustrated, e.g., by the concentration exponents determined by Withell (1942) for phenol and *p*-chloro-*m*-cresol respectively, viz., 4.55 and 8.3.

At any rate, the antibacterial action of phenol will be referred to repeatedly in the discussion of the various phenol derivatives, the former serving as the basis of a quantitative expression of the antibacterial effectiveness of the latter. The methods of determining this quantitative relationship and the definitions of the relevant terms (e.g., "phenol coefficient") are given in another chapter.

## GERMICIDAL ACTION OF SIMPLE PHENOL HOMOLOGS

Table 72 gives the phenol coefficients of a series of simple phenol homologs obtained (at  $37^{\circ}$ C) with *Salmonella typhosa*, *Micrococcus pyogenes* var. *aureus*, *Mycobacterium tuberculosis* (hom.), and *Candida albicans* as test organisms (Klarmann and



Shternov, 1936). It appears that in this series the germicidal potency with regard to all four test organisms increases with the increasing molecular weight until the *n*-amyl derivative is reached; beginning with this point, a further increase of the molecular weight produces a very considerable increase of the germicidal action upon *M. pyogenes* var. *aureus*, *M. tuberculosis* (hom.), and *C. albicans*, while with respect to *S. typhosa* the effectiveness declines, becoming rather indefinite in the case of the heptyl phenol which nevertheless shows considerable activity against the other three test organisms. The figures indicate that, in the case of the compounds up to and including *n*-butyl phenol, the *S. typhosa* phenol coefficient might serve as a reasonably accurate index of germicidal potency, since a compound which is, e.g., 2, 6, 18 or 45 times respectively more effective than phenol against *S. typhosa*, is also approximately as many times more effective against the other microorganisms studied. However, beginning with the *n*-amyl derivatives, this relation ceases to exist; in this case as well as in that of the higher homologs, the *S. typhosa* phenol coefficient is no longer indicative of the general germicidal efficacy.

With specific reference to coal tar disinfectants, attention is called to the results obtained with the pure cresols and xylenols (methyl and dimethyl phenols) given in Table 72 and also in Table 73 in which the parallelism in the germicidal effects upon the different microorganisms is distinctly evident. These results are significant because cresols and xylenols occur in the usual run of such disinfectants. (The experimental conditions of the tests described in Tables 72 and 73 differ in that in the former case the temperature at which the culture was exposed to the action of the disinfectant solution was 37°C, in the latter 20°C).

It may be mentioned in passing that the term "quasi-specific" has been proposed (Klarmann, Shternov and Gates, 1934) to describe the effect such as that shown, e.g., by *n*-heptyl phenol which is extremely active against *M. pyogenes* var. *aureus* and yet very little effective against *S. typhosa*.

The bactericidal action of the homologous *p*-alkyl phenol derivatives has been studied by other investigators. Coulthard, Marshall and Pyman (1930) observed an increasing potency with respect to *S. typhosa* resulting from the lengthening of the substituting alkyl radical up to the amyl, and a subsequent drop of this potency in the case of the hexyl and heptyl radicals. The phenol coefficients of *n*-amyl cresol have been reported by Coulthard and Pyman (1931) as follows: against *S. typhosa* 250, *E. coli* 200, *M. pyogenes* var. *aureus* 220, *S. faecalis* 200. Inhibitory action was observed in dilutions of 1:10,000 to 1:20,000.

According to Rettger, Valley and Plastridge (1929), the three isomers of butyl phenol show approximately the same germicidal action, the phenol coefficients being 51 for the *o*- and *p*-butyl phenols and 52 for the *n*-butyl derivative. On the other hand, Bartz, Miller and Adams (1935) found the introduction of the isobutyl radical into the nucleus of phenol less effective in enhancing the antibacterial power than that of the *n*-butyl or of a higher alkyl radical. Of several isobutyl derivatives prepared, the highest germicidal power was displayed by 2-isobutyl-4,5-dimethyl phenol which was found to kill *M. pyogenes* var. *aureus* in a dilution of 1:5,000.

For additional data on alkyl phenols and their antimicrobial potency reference may be made to the papers by Read and Mullin (1928), Read and Miller (1932), Schaffer and Tilley (1926, 1927), and Woodward, Kingery and Williams (1933).

### BIS(HYDROXYPHENYL) ALKANES

Because of their chemical configuration the group of *bis*-(hydroxyphenyl) alkanes justifies classification and discussion under the general heading of phenol derivatives. Harden and Reid (1932), and subsequently Richardson and Reid (1940) studied two groups of compounds of the types:  $\text{HOC}_6\text{H}_4\text{CH(R)C}_6\text{H}_4\text{OH}$  and  $\text{HOC}_6\text{H}_4(\text{CH}_2)_n\text{C}_6\text{H}_4\text{OH}$ . In both groups the hydroxyl is in para position. In the former R may be hydrogen or an alkyl group from methyl to *n*-hexyl; in the latter *n* may vary from 1 to 10. Increasing the length of the aliphatic chain in the *alpha,alpha*-group resulted in an increase of the germicidal effect upon *M. pyogenes* var. *aureus*; in the *alpha,omega*-group maximum killing effects upon the same test organism were observed only in the case of the first four members of the series.

A large number of *bis*-(hydroxyphenyl) alkanes were studied by Heinemann (1944). These compounds derived from *bis*-(hydroxyphenyl) propane are of the type  $\text{HOC}_6\text{H}_4\text{CH(R}_1\text{)CH(R}_2\text{)-CH(R}_3\text{)C}_6\text{H}_4\text{OH}$  where *R*<sub>1</sub>, *R*<sub>2</sub> and *R*<sub>3</sub> may be hydrogen or an organic radical. Another variant is introduced by the relative position of the hydroxyl radicals in both benzene rings. In substantial agreement with the findings obtained in other cases, the following was found in the case of these compounds:

- (1) Increasing the length of the aliphatic chain attached to a hydroxyphenyl ring results in increased antibacterial activity.
- (2) A given number of carbon atoms contributes a greater effectiveness in a single chain than when distributed between two or more chains.

TABLE 73.—BACTERICIDAL ACTION OF CRESOLS AND XYLENOLS AT 20° C.

	<i>Salmonella typhosa</i>		<i>Shigella paradysenteriae</i> (Flexner)		<i>Micrococcus pyogenes</i> var. <i>aureus</i>		<i>Streptococcus pyogenes</i> (hemol.)		<i>Mycobacterium tuberculosis</i>	
	Concn.*	Phenol coeff.	Concn.*	Phenol coeff.	Concn.*	Phenol coeff.	Concn.*	Phenol coeff.	Concn.*	Phenol coeff.
Phenol (I)	1:80-90	1.0	1:90-100	1.0	1:60	1.0	1:60	1.0	1:60	1.0
2-Methyl I	1:200	2.5	1:250	2.5	1:130	2.2	1:150	2.5	1:160	2.7
3-Methyl I	1:200	2.5	1:200	2.0	1:140	2.3	1:140	2.3	1:140	2.3
4-Methyl I	1:200	2.5	1:200	2.0	1:130	2.2	1:140	2.3	1:140	2.3
2,5-Dimethyl I	1:400	5.0	1:500	5.5	1:250	4.2	1:250	4.2	1:300	5.0
2,4-Dimethyl I	1:400	5.0	1:500	5.5	1:250	4.2	1:300	5.0	1:350	5.0
3,4-Dimethyl I	1:400	5.0	1:500	5.5	1:250	4.2	1:250	4.2	1:300	5.0
2,6-Dimethyl I	1:400	5.0	1:500	5.5	1:250	4.2	1:300	5.0	1:300	5.0

\* Minimum concentrations effective in 10 minutes.



(3) Branching of the aliphatic chain lowers the activity below that of the normal straight chain compound.

(4) The position of the hydroxyl group on the rings does not materially influence bactericidal activity in unsubstituted isomeric compounds.

(5) The "quasi-specific" character of the compounds is in evidence; thus the susceptibility of *S. typhosa* reaches a maximum with an alkyl chain containing four carbon atoms whereas homologs of higher molecular weight, up to an alkyl chain of nine carbon atoms, are quite effective against *M. pyogenes* var. *aureus*.

In a group of 23 compounds only 3 were found to be active against *S. typhosa*, the most effective being 1,3-bis(*p*-hydroxyphenyl) butane which was bactericidal in a dilution of 1:8000. With respect to *M. pyogenes* var. *aureus* the most effective homolog is 1,3-bis(*p*-hydroxyphenyl) octane with a bactericidal dilution of 1:150,000. Less effective but still quite potent is *e.g.*, 4,6-bis(*p*-hydroxyphenyl) nonane with a bactericidal dilution of 1:90,000.

The functional variants of diethyl stilbestrol investigated by Rubin and Wishinsky (1944) belong in this chapter. While diethyl stilbestrol itself is inhibitory to *M. pyogenes* var. *aureus* in a dilution of less than 1:10,000, the monohydroxylated, *i.e.*, phenolic derivative  $C_6H_5C(C_2H_5)=C(C_2H_5)C_6H_4OH$  inhibits multiplication of this organism in a dilution of 1:640,000, and its monobromoderivative does it in one of 1:1,000,000. This is in general agreement with the information available for substituted phenol derivatives.

## TECHNICAL COAL TAR DISINFECTANTS

While phenol and the three cresols are slightly soluble in water, the higher homologs display a decreasing solubility with the increasing weight of their substituting radicals; other things being equal this solubility depends also upon the structure of the substituent, *i.e.*, whether it is a normal, or a branched chain alkyl radical, or a cycloalkyl, aralkyl, aryl radical, etc.

In the case of the poorly soluble phenol homologs and also in that of other phenol derivatives of low solubility, advantage is taken often of the "solubilizing" effect of soaps when it is desired to obtain easy and rapid miscibility with water. However, considerable care must be exercised in arriving at the optimal ratio of soap to phenol derivative; a deficiency of soap will affect the stability of the "solution" while an excess of soap will act like a "lipoid phase" tending to reduce the proportion of the phenolic compound available for antibacterial action. Indeed the antibacterial effect in this class of compounds may be said to depend, among other things,

upon the tendency of the phenolic agent to migrate from the aqueous to the "lipoid" phase; the latter is represented in the absence of any interfering factors by the totality of the bacterial cells exposed to the action of the particular phenolic compound. Where an interfering factor is present, *e.g.*, in the form of another definite lipid phase (oil), or of an added water miscible solvent (alcohol, ethylene glycol, glycerol), or of an excessive proportion of soap (also of certain other emulsifying agents), a minor or major proportion of the phenolic compound is rendered unavailable for antibacterial action owing to its engagement by such an interfering factor (Tilley and Schaffer, 1931; Gershenfeld and Miller, 1933; Cooper, 1947, 1948, 1949).

Incidentally, in the case of the soap effect, not only the proportion but also the composition of soap plays a determining role. Thus in compounding disinfectants of the type of Saponated Cresol Solution, coconut oil soap used in the correct proportion appears to enhance the antibacterial effect of cresol (against *S. typhosa* though not against *M. pyogenes* var. *aureus*) while soaps derived from linseed oil or soybean oil have no such action (Tilley and Schaffer, 1925). In any case, a substantial excess of soap may deprive the phenol derivative of most, if not all, of its germicidal power (Tilley and Schaffer, 1930).

The appearance on the market of "synthetic" phenolic disinfectants is a comparatively recent phenomenon. Prior to this the phenolic disinfectants employed in the practice of disinfection were almost exclusively of the category of coal-tar disinfectants. Within this category there are two different groups of disinfectants, each with very definite characteristics, *viz.*, the "soluble" and the "emulsifiable" group. The former comprises preparations containing as active principle phenol derivatives of low molecular weight, such as the isomeric cresols or xylenols; the Saponated Cresol Solution of the National Formulary (formerly of the U. S. Pharmacopeia) is the outstanding example of this class. The products of the latter group contain, in addition to phenolic constituents, varying proportions of coal-tar hydrocarbons and other constituents of the so-called "neutral oil." Soaps made from vegetable oils or resins form an essential part of the formulas of both groups, their miscibility with water being due to the presence of soap. Physically, the two classes may be distinguished by the appearance of their mixtures with distilled water; in low concentrations, the "soluble" disinfectants form practically clear solutions while those of the "emulsifiable" group show a milky turbidity.

The regularities in antibacterial behavior referred to in the case of the lower homologs of the alkyl phenol series are observed

also with the usual variety of the "soluble" coal-tar disinfectants which as a rule contain the cresol or the xylenol isomers; here the *S. typhosa* phenol coefficient may be expected to give a reasonable idea of their general germicidal potency. This is borne out by the results obtained with three commercial "soluble" disinfectants of different germicidal strengths (Cresol Compound N.F. and Cresylic disinfectants A and B), listed in Table 74. (Klarmann and Shternov, 1936)

This table gives also the results obtained with six commercial "emulsifiable" tar oil disinfectants, and here the picture is entirely different. Although the preparations are listed in the order of their *S. typhosa* phenol coefficients, beginning with the lowest figure, there appears to be no relationship between these figures and the phenol coefficients obtained with the other microorganisms. By way of comparison with the three disinfectants of the "soluble" group, where, *e.g.*, the ratio of the *S. typhosa* and *Streptococcus* phenol coefficients is not in excess of approximately 2 to 1 for any given product, in the "emulsifiable" group there occur such ratios as 8 to 1 or even 20 to 1.

A comparison of the germicidal effects upon *S. typhosa* and *Streptococcus pyogenes* (*hemol.*) of a number of technical tar-oil and cresylic disinfectants was carried out by Brewer and Ruehle (1931). In contrast to Philbrick (1929) who reported upon this subject previously, the findings of Brewer and Ruehle substantiate the contention that the *S. typhosa* phenol coefficient is unable to furnish an adequate description of the germicidal potency of all coal-tar disinfectants. These authors list certain products whose comparatively high *S. typhosa* phenol coefficients of over 3 would conceal the fact that they are from about 10 to 30 times less effective against *S. pyogenes* (*hemol.*), thus tending to misrepresent their germicidal value.

As indicated above, the emulsifiable coal-tar disinfectants contain varying proportions of "neutral oil"; this consists mostly of methyl and dimethyl naphthalenes, besides other hydrocarbons (fluorene, acenaphthene), organic bases (quinoline, pyridine) and their alkyl derivatives, certain oxygenated compounds, organic sulfur compounds, etc. Neutral oil itself is practically free from phenol or phenol derivatives. Obviously, therefore, the antibacterial action of disinfectants containing considerable proportions of neutral oil hydrocarbons cannot be expected to show the functional regularity encountered in the case of phenolic compounds.

There is additional experimental information relevant to this matter. Klarmann and Shternov (1936) found that gradual replacement by neutral oil of cresol does not reduce the *S. typhosa*



TABLE 74.—BACTERICIDAL ACTION OF CERTAIN "SOLUBLE" AND "EMULSIFIABLE" DISINFECTANTS AT 20° C.

	<i>Salmonella typhosa</i>		<i>Shigella paradysenteriae</i> (Flemer)		<i>Micrococcus pyogenes</i> var. <i>aureus</i>		<i>Streptococcus pyogenes</i> (hemol.)		<i>Mycobacterium tuberculosis</i>	
	Concn.*	Phenol coeff.	Concn.*	Phenol coeff.	Concn.*	Phenol coeff.	Concn.*	Phenol coeff.	Concn.*	Phenol coeff.
Cresol Compound, NF	1:200	2.3	1:200	2.2	1:100	1.6	1:100	1.4	1:140	2.3
Cresylic disinfectant, A	1:300	3.8	1:400	4.4	1:120	2.4	1:180	2.6	1:200	3.3
Cresylic disinfectant, B	1:500	5.5	1:500	5.5	1:180	3.0	1:200	2.9	1:300	5.0
Tar-Oil disinfectant, A	1:300	2.8	1:350	3.9	1:40	0.7	1:50	0.7	1:400	2.3
Tar-Oil disinfectant, B	1:600	6.7	1:600	6.7	1:25	0.4	1:25	0.4	1:160	2.7
Tar-Oil disinfectant, C	1:700	7.8	1:500	5.5	1:25	0.4	1:25	0.4	1:140	2.3
Tar-Oil disinfectant, D	1:700	8.8	1:600	6.7	1:40	0.7	1:70	1.0	1:160	2.7
Tar-Oil disinfectant, E	1:800	8.8	1:1000	11.1	1:100	1.7	1:120	1.7	1:80	1.3
Tar-Oil disinfectant, F	1:1000	11.1	1:1000	11.1	1:120	2.0	1:120	1.7	1:250	4.2
Phenol (control)	1:80-90	1.0	1:90-100	1.0	1:60	1.0	1:60-70	1.0	1:60	1.0

\* Minimum concentrations effective in 10 minutes.

phenol coefficient of an emulsifiable disinfectant formulated with such a mixture; on the other hand the effect upon *M. pyogenes* var. *aureus*, *S. pyogenes* (hemol.) and *M. tuberculosis* (hom.) decreases as the proportion of cresol goes down and that of the neutral oil goes up. Substantially the same results were obtained when the replacement of cresol was made respectively with alpha- and beta-methyl naphthalene.

Thus although the *S. typhosa* phenol coefficient is used extensively as an index of the germicidal potency of coal-tar and related disinfectants, it seems proper to call attention to its limitations in the light of the above experimental data. (Incidentally, similar limitations apply to other classes of disinfectants bearing no relation to phenol, e.g., pine oil compounds, quaternary ammonium salts, etc.). As indicated above, in the case of the pure alkyl phenol derivatives, the germicidal effect upon *S. typhosa* of the lower homologs parallels that upon other vegetative pathogenic microorganisms to such a degree that the *S. typhosa* phenol coefficient might be regarded as a relative index of the germicidal potency of these compounds. Since "soluble" coal-tar disinfectants contain certain lower phenol homologs as their active principle, the same consideration would apply in their case.

However, in the case of the "emulsifiable" disinfectants, there exists no quantitative relationship between the effect upon *S. typhosa* and that upon other microorganisms; thus some disinfectants of this type, with high *S. typhosa* phenol coefficients, may be actually less effective against other pathogenic microorganisms than others with lower *S. typhosa* coefficients; or of two products with the same phenol coefficient, one may be a much better general germicide than the other. This condition arises mainly from the indefiniteness of the composition of the "emulsifiable" disinfectants, and its quantitative expression is affected particularly by the variability in the ratio of phenolic to non-phenolic constituents.

## PETROLEUM AND OTHER SOURCES OF PHENOLIC MATERIAL

A newer source of phenolic materials suitable for use in the formulation of disinfectants is petroleum. Phenolic compounds occur in petroleum distillates primarily by reason of certain thermal alterations such as would take place in the course of cracking. It has been known for some time that phenol, the three cresols—certain xyenol isomers, di- and triethyl phenols, and beta-naphthol occur in crude petroleum distillates of various origins. Field, Dempster and Tilson (1940) identified the following phenol derivatives:

in the various distillation cuts: phenol, *o*-, *m*-, and *p*-cresol, 1,4,2-, 1,3,4-, 1,2,3-, 1,3,5- and 1,2,4-xylenols, and isopseudocumenol. The phenol coefficients of several "petroleum acid" fractions are given in the following table 75.

TABLE 75.—PHENOL COEFFICIENTS OF "PETROLEUM ACIDS"

Fraction in ° C.	Phenol coefficient
200-203	4.3
212-215	5.9
224-227	11.6
236-239	18.8
248-251	15.3
260-263	16.5

By way of comparison, the tar acid fraction of 224 to 227°C gives a phenol coefficient of 9.5, *i.e.*, one slightly lower than that of the corresponding petroleum acid fraction.

While sporadic attempts have been made to utilize phenolic compounds from petroleum, no significant position appears to have been gained to date by this source of primary material for the production of disinfectants.

Very recently another technical source of alkyl phenol derivatives has been opened up, *viz.*, that of hydrogenation of coal. Although no information is available as yet as to the fitness of this material for disinfectant formulation, one would be justified in assuming that it should be useful for this purpose. The principal constituents are *m*-dialkyl phenols; the total number of carbon atoms in both alkyl radicals varies from three to five. The boiling range of the mixture is 230° to 270°C.

## HALOGENATED PHENOL DERIVATIVES

That halogenation of phenolic compounds leads to a potentiation of their antibacterial effectiveness has been known for a long time. As early as 1906 (Bechhold and Ehrlich), it was observed that polychloro- and polybromoderivatives of phenol and of betanaphthol were possessed of considerable effectiveness against *Corynebacterium diphtheriae* and *M. pyogenes* var. *aureus*; also that this effectiveness was not retained in the presence of serum; moreover, the polyhalogen compounds displayed considerable toxicity (Bechhold, 1909, 1914).

Laubenheimer (1909) showed that monohalogen substituted alkyl derivatives of phenol, while considerably more active than the unsubstituted compounds, were only partly inactivated in the presence of serum.



More recently, Wolf and Westveer (1952) showed that in the case of phenol itself potentiation by chlorine substitution occurs only until the trichloro-derivatives have been reached; the tetrachloro-isomers are considerably less active than any of the trichloro-isomers, and pentachlorophenol is about as effective as the phenol itself against *S. typhosa* although more effective than the latter against *M. pyogenes* var. *aureus*. There is considerable difference in the germicidal activity between five (of six possible) trichlorophenol isomers tested; thus at a constant pH 6.7, the killing dilution for *S. typhosa* of 2,3,6-trichlorophenol is 1:500, that of the 2,4,5-isomer 1:4200. On the other hand the activity of the trichlorophenols depends to an extraordinary degree upon pH variations; thus 2,3,4-trichlorophenol kills *S. typhosa* at pH 7.2 in 1:2400, but at pH 8.2 only in the 8 times stronger concentration of 1:300. Similar responses were obtained with the other trichloro-isomers.

The influence of a stepwise substitution by halogen in the phenol nucleus upon bactericidal effectiveness is referred to also in a paper by Klarmann, Shternov, and Von Wowern (1929) showing that the impairment of this effectiveness by organic matter too, is a function of the extent of the halogen substitution. The mono-substituted phenol derivatives suffer a lower reduction, comparable to that of phenol itself, whereas the loss of potency in the presence of organic matter shown, *e.g.*, by 2,4,6-trichlorophenol, is of the order of 50 per cent with respect to both *S. typhosa* and *M. pyogenes* var. *aureus*. It should be emphasized at this point that in almost all studies dealing with the effect of nucleus substitution upon antibacterial action of phenol derivatives the quantitative data reported by the various investigators need not be regarded necessarily as indicative of the practical germicidal effectiveness of the compounds studied. As a rule, these data are mostly of a theoretical interest in that they illustrate the relationships between the chemical structure of the phenol derivatives and their antibacterial *potential*; they do not usually assure one as to a given compound's superiority or even fitness for *practical* use, *i.e.*, in the presence of one or more of the interfering factors encountered in the practice of disinfection or antisepsis, such as organic and inorganic soil, pus, serum, blood, mucus, dejecta, etc.

A systematic examination of the relationships between the chemical structure and the microbicidal action of aliphatic and aromatic substitution derivatives of *p*- and *o*-chlorophenol has been carried out by Klarmann, Gates, Shternov and Cox (1933). The following tables 76 and 77 illustrate the conditions found in the case of ortho-alkyl derivatives of *p*-chlorophenol and of para-

TABLE 76. — MICROBICIDAL ACTION OF ORTHOALKYL DERIVATIVES OF PARACHLOROPHENOL (PHENOL COEFFICIENTS, 37° C.)

Name	<i>Salmonella typhosa</i>	<i>Salmonella schott-muelleri</i>	<i>Micrococcus pyogenes var. aureus</i>	<i>Streptococcus pyogenes (hemol.)</i>	<i>Mycobacterium tuberculosis</i>	<i>Trichophyton schoenleinii</i>	<i>Candida albicans</i>
<i>p</i> -Chlorophenol (II)	4.3	4.3	4.3	4.4	3.9	3.3	4.0
Methyl II	12.5	12.9	12.5	11.1	11.1	7.1	11.1
Ethyl II	28.6	28.6	34.4	31.3	27.8	25.0	32.5
<i>n</i> -Propyl II	93.3	714.0	93.8	77.8	66.7	714.0	100.0
<i>n</i> -Butyl II	141.0	114.0	257.0	250.0	178.0	156.0	178.0
<i>n</i> -Amyl II	156.0	100.0	500.0	556.0	389.0	278.0	389.0
<i>sec</i> -Amyl II	46.7	42.9	312.0	312.0	222.0	229.0	182.0
<i>n</i> -Hexyl II	(23.2)	(21.4)	1250.0	1333.0	333.0	357.0	556.0
<i>Cyclo</i> Hexyl II	<26.7	<14.3	438.0	361.0	278.0	222.0	300.0
<i>n</i> -Heptyl II	(20.0)	(14.3)	1500.0	2222.0	>400.0	175.0	>363.0
<i>n</i> -Octyl II	—	—	1750.0	>312.0	—	—	—

Figures in parentheses approximate

TABLE 77. — MICROBICIDAL ACTION OF PARAALKYL DERIVATIVES OF ORTHOCHLOROPHENOL (PHENOL COEFFICIENTS, 37° C.)

Name	<i>Salmonella typhosa</i>	<i>Salmonella schott-muelleri</i>	<i>Micrococcus pyogenes var. aureus</i>	<i>Streptococcus pyogenes (hemol.)</i>	<i>Mycobacterium tuberculosis</i>	<i>Candida albicans</i>
<i>o</i> -Chlorophenol (III)	2.5	2.1	2.9	2.0	2.2	2.2
Methyl III	6.3	5.4	7.5	5.6	5.6	8.3
Ethyl III	17.2	25.0	15.7	15.0	17.8	22.2
<i>n</i> -Propyl III	40.0	35.7	32.1	33.3	33.3	44.4
<i>n</i> -Butyl III	86.7	66.7	93.8	88.9	77.8	88.9
<i>n</i> -Amyl III	80.0	40.0	286.0	222.0	222.0	278.0
<i>tert</i> Amyl III	32.1	21.4	125.0	122.0	111.0	100.0
<i>n</i> -Hexyl III	23.3	—	500.0	555.0	178.0	278.0
<i>n</i> -Heptyl III	16.7	—	375.0	350.0	77.8	70.0

alkyl derivatives of *o*-chlorophenol; tables 78 and 79 deal with ortho-alkyl derivatives of *p*-bromophenol and with para-alkyl deri-

TABLE 78. — MICROBICIDAL ACTION OF ORTHOALKYL DERIVATIVES OF PARABROMOPHENOL (PHENOL COEFFICIENTS, 37° C.)

	<i>Salmonella typhosa</i>	<i>Micrococcus pyogenes</i> <i>var. aureus</i>	<i>Mycobacterium tuberculosis</i>	<i>Candida albicans</i>
<i>p</i> -Bromophenol (IV)	6.0	5.0	5.6	6.3
Methyl IV	12.5	11.3	13.3	13.3
Ethyl IV	31.3	25.0	27.8	27.8
<i>n</i> -Propyl IV	62.5	62.5	77.8	77.8
<i>n</i> -Butyl IV	156.0	313.0	278.0	222.0
<i>n</i> -Amyl VI	62.5	571.0	444.0	278.0
<i>sec.</i> Amyl IV	> 33	150.0	156.0	150.0
<i>n</i> -Hexyl IV	—	1250.0	778.0	278.0
<i>cyclo</i> Hexyl IV	> 23	429.0	278.0	222.0

TABLE 79. — MICROBICIDAL ACTION OF PARAALKYL DERIVATIVES OF ORTHOBROMOPHENOL (PHENOL COEFFICIENTS, 37° C.)

Name	<i>Salmonella typhosa</i>	<i>Micrococcus pyogenes</i> <i>var. aureus</i>	<i>Mycobacterium tuberculosis</i>	<i>Candida albicans</i>
<i>o</i> -Bromophenol (V)	3.3	3.1	3.9	3.8
<i>tert</i> Amyl V	33.3	150.0	100.0	77.8
<i>n</i> -Hexyl V	> 20.0	625.0	556.0	222.0
<i>n</i> -Propyl- <i>m,m</i> -dimethyl V	—	357.0	220.0	138.0

vatives of *o*-bromophenol respectively. The regularities encountered may be summarized as follows:

(1) Halogen substitution intensifies the microbicidal potency of phenol derivatives, the presence of halogen in the para-position to the hydroxyl group being more effective in this respect than in the ortho-position.

(2) Introduction of aliphatic or aromatic groups into the nucleus of halogen phenols increases the bactericidal potency (up to certain limits), this increase depending in the case of alkyl substitution upon the number of carbon atoms present in the substituting group or groups.

(3) As a rule the intensifying effect upon the bactericidal potency of a normal aliphatic chain with a given number of carbon atoms is greater than that of a branched chain, or of two alkyl groups with the same total number of carbon atoms.

(4) Ortho-alkyl derivatives of *p*-chlorophenol are more active-ly germicidal than para-alkyl derivatives of *o*-chlorophenol.

(5) In the case of the higher homologs the germicidal action manifests a "quasi-specific" character in that, beginning with a definite point (which is different in the case of the various test organisms), a further increase in the weight of the substituting



groups causes the germicidal capacity to drop sometimes to almost total inactivity with respect to certain microorganisms, (*e.g.*, *S. typhosa*), and at the same time to rise to comparatively enormous values with respect to others (*e.g.*, *M. pyogenes* var. *aureus*).

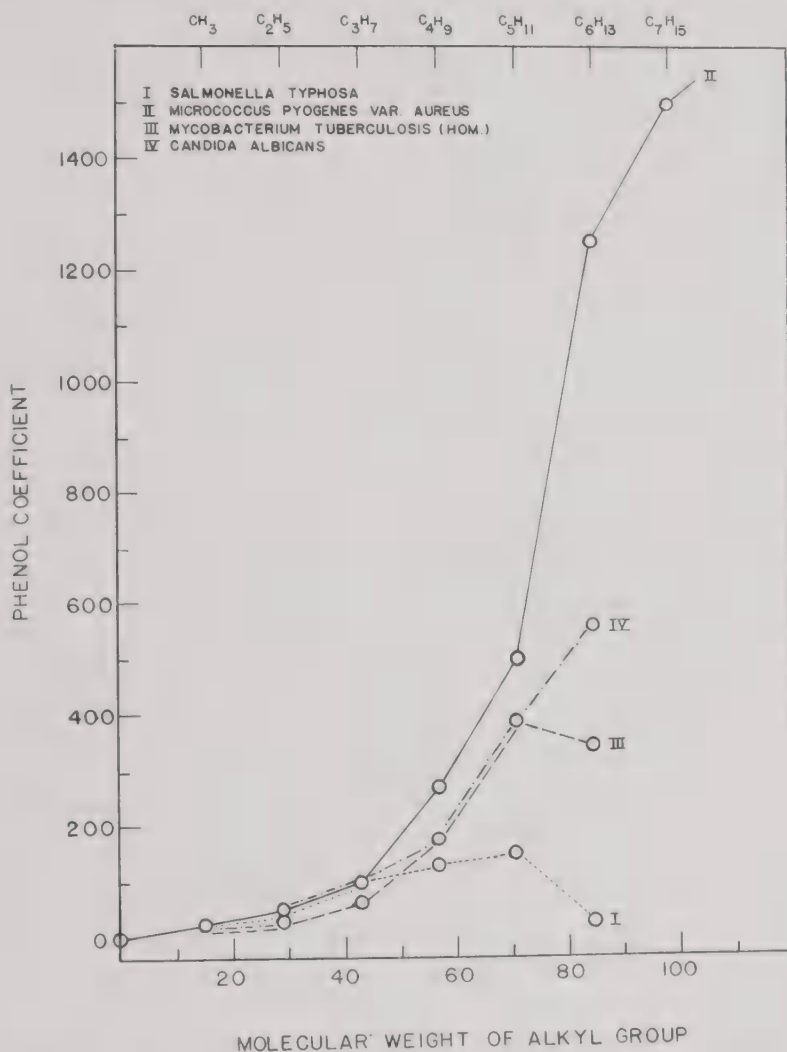


Fig. 16.—The “Quasi-specific” Effect in the Case of the Homologous Series of *o*-Alkyl *p*-Chlorophenol Derivatives.

A visual illustration of this “quasi-specific” behavior is supplied by Figure 16 which also indicates the difference in the order of magnitude of the bactericidal potencies with respect to the several groups of microorganisms under discussion.

In addition to the homologous series of alkyl derivatives of *p*- and *o*-chlorophenol, and of *p*- and *o*-bromophenol, a series of

polyalkyl and of aromatic derivatives of *p*-chlorophenol were studied for their microbicidal potency. The former are referred to in Table 80, the latter in Table 81.

TABLE 80.—BACTERICIDAL ACTION OF POLYALKYL DERIVATIVES OF PARACHLOROPHENOL  
(PHENOL COEFFICIENTS, 37° C.)

Name	<i>Salmonella</i> <i>typhosa</i>	<i>Micrococcus</i> <i>pyogenes</i> var. <i>aureus</i>	<i>Streptococcus</i> <i>pyogenes</i> (hemol.)	<i>Mycobacterium</i> <i>smegmatis</i>
<i>p</i> -Chlorophenol (II)	4 3	4 3	4 4	3 9
3-Methyl II	10 7	11 3	11 3	11 1
3,5-Dimethyl II	30 0	25 7	27 5	28 1
6-Ethyl-3-methyl II	64 3	50 0	55 6	55 6
6- <i>n</i> -Propyl-3-methyl II	133 0	200 0	178 0	156 0
6- <i>iso</i> -Propyl-3-methyl II	107 0	150 0	138 0	138 0
2-Ethyl-3,5-dimethyl II	46 4	106 0	94 4	122 0
6- <i>sec</i> Butyl-3-methyl II	50 0	500 0	361 0	389 0
2- <i>iso</i> -Propyl-3,5-dimethyl II	81 3	313 0	313 0	325 0
6-Diethylmethyl-3-methyl II	23 3	625 0	611 0	777 0
6- <i>iso</i> -Propyl-2-ethyl-3-methyl II	56 7	200 0	175 0	200 0
2- <i>sec</i> Butyl-3,5-dimethyl II	28 6	563 0	556 0	556 0
2- <i>sec</i> Amyl-3,5-dimethyl II	(15 6)	750 0	889 0	700 0
2-Diethylmethyl-3,5-dimethyl II	<13 0	1143 0	1000 0	667 0
6- <i>sec</i> Octyl-3-methyl II	(21 4)	>89 0	122 0	>70 0

Figures in parentheses approximate

TABLE 81.—BACTERICIDAL ACTION OF AROMATIC DERIVATIVES OF PARACHLOROPHENOL  
(PHENOL COEFFICIENTS, 37° C.)

Name	<i>Salmonella</i> <i>typhosa</i>	<i>Salmonella</i> <i>schottmuelleri</i>	<i>Micrococcus</i> <i>pyogenes</i> var. <i>aureus</i>	<i>Streptococcus</i> <i>pyogenes</i> (hemol.)	<i>Mycobacterium</i> <i>tuberculosis</i>
<i>p</i> -Chlorophenol (II)	4 3	4 3	4 3	4 4	4 0
<i>o</i> -Benzyl II	71 4	71 4	200 0	225 0	178 0
<i>o</i> -Benzyl- <i>m</i> -methyl II	18 3	28 6	375 0	389 0	—
<i>o</i> -Benzyl- <i>m</i> , <i>m</i> -dimethyl II	—	—	750 0	778 0	—
<i>o</i> -Phenylethyl II	100 0	71 4	375 0	500 0	333 0
<i>o</i> -Phenylethyl- <i>m</i> -methyl II	—	—	375 0	250 0	178 0

Here again, as in the monoalkyl series, the weight of the substituting groups determines the selective "quasi-specific" action.

As to the effect upon the germicidal action of the distribution of the added weight over several substituting radicals, a comparison, *e.g.*, of the substituted *p*-chlorophenol derivatives with a total of four substituting carbon atoms will furnish the necessary illustration. It shows that substitution of *p*-chlorophenol by one alkyl group generally leads to a more effective compound than substitution by two or three alkyl groups with the same total number of carbon atoms; similarly, dialkyl substituted derivatives are considerably more germicidal than trialkyl substituted ones.

It is found in this group, too, that isoalkyl substituted compounds are less effective than the corresponding normal alkyl derivatives. This follows from the comparison of 6-*n*-propyl-3-

methyl-4-chlorophenol and 6-isopropyl-3-methyl-4-chlorophenol (chlorothymol); thus the *n*-propyl derivative is distinctly more potent than chlorothymol.

Polyalkyl compounds of equal molecular weight, containing isoalkyl groups, may show considerable variation in their germicidal action, depending upon their structure.

Whereas in the case of the normal monoalkyl derivatives the maximum effect upon *S. typhosa* was shown by a compound with 5 carbon atoms in the side chain, in the case of the polyalkyl derivatives this maximum is reached by a compound with a total of 4 substituting carbon atoms. As to the other test organisms in Table 80, the derivatives with a total of 7 carbon atoms generally show the greatest microbicidal efficacy.

The effect of steric isomerism is illustrated by the findings of Heicken (1939). This author found the chlorine and bromine derivatives of the 1,2,3-, 1,3,5-, and 1,4,2-xylenols to be 50 to 70 times as effective as phenol (against *E. coli* and *M. pyogenes* var. *aureus*) while those of the 1,2,4-, 1,3,2- and 1,3,4-xylenols showed an effectiveness only 15 to 20 times as great. These studies were extended by Lockemann and Heicken (1939).

Also among the aromatic derivatives of *p*-chlorophenol there are some potent germicides (Klarmann, Gates and Shternov 1932*b*). As to the effect of their chemical constitution upon germicidal action, it is noteworthy that the step from the benzyl derivative to the next one with a higher molecular weight is accompanied by different results depending upon the place in the molecular structure to which the additional weight is attached. Thus, 5-methyl-2-benzyl-4-chlorophenol is much less effective than 2-benzyl-4-chlorophenol against the four test organisms of the typhoid-colon group, while 2-phenylethyl-4-chlorophenol is very much more effective than the former. With reference to the other organisms, the difference in the germicidal potencies of the methyl-benzyl and the phenylethyl derivatives is less pronounced.

A further increase in the molecular weight lowers the germicidal action with reference to the bacteria of the typhoid-colon group as shown by the figures obtained with 3,5-dimethyl-2-benzyl-4-chlorophenol. However the germicidal potency is increased considerably with regard to the other four organisms. Curiously, 3-methyl-6-phenylethyl-4-chlorophenol which is isomeric with 3,5-dimethyl-2-benzyl-4-chlorophenol, is not only less effective than the latter, but also, in most instances, weaker than the 2-phenylethyl derivative.

The aliphatic and aromatic derivatives of the chloro- and bromophenols studied display a noteworthy bacteriostatic action.



This is illustrated by the following results (Table 82) obtained respectively with *o-n*-amyl and with *o-n*-heptyl *p*-chlorophenol.

For the sake of possible theoretical interest, mention may be made here of the series of 2-alkyl-4-fluorophenol derivatives prepared by Suter, Lawson, and Smith (1939). The following phenol coefficients were determined for the individual members of this

TABLE 82.—BACTERIOSTATIC DILUTIONS OF ORTHO-*n*-AMYL AND ORTHO-*n*-HEPTYL PARACHLOROPHENOL

Name	<i>Micrococcus</i> <i>pyogenes</i>	<i>Mycobacterium</i> <i>smegmatis</i>	<i>Mycobacterium</i> <i>tuberculosis</i>
	<i>var. aureus</i>		
<i>n</i> -Amyl II	1:150,000	1:200,000	1:150,000
<i>n</i> -Heptyl II	1:200,000	1:200,000	1:200,000

series: ethyl 10, propyl 21, butyl 66, amyl 69, hexyl less than 62.

It is significant that the increase in the molecular weight of the phenol derivatives which is accompanied by an increase in antibacterial potency is also accompanied by decreasing toxicity. This is shown by the results given in the following Table 83 which states the fatal doses in milligrams per gram obtained upon subcutaneous injection in mice (Klarmann, Shternov and Gates, 1934).

TABLE 83.—TOXICOLOGICAL DATA—MILLIGRAMS PER GRAM, SUBCUTANEOUS INJECTION IN MICE

Phenol Parachlorophenol Orthochlorophenol	0.45	0.6	0.7
	Para-alkyl derivatives of phenol	Ortho-alkyl derivatives of parachlorophenol	Para-alkyl derivatives of ortho-chlorophenol
Methyl	0.5	2.0	1.5
Ethyl	1.0	4.0	4.0
<i>n</i> -Propyl	2.0	6.0	6.0
<i>n</i> -Butyl	3.0	15.0	15.0
<i>n</i> -Amyl	5.0	>20.0	20.0
<i>n</i> -Hexyl	6.0	>20.0	>20.0
<i>n</i> -Heptyl	10.0	>20.0	>20.0

### INDIVIDUAL PHENOL DERIVATIVES IN PRACTICAL APPLICATION

For the sake of completeness, brief reference may be made to a number of phenol derivatives which, for one reason or another, have gained some practical prominence.

According to Schaffer and Tilley (1927), thymol and carvacrol show comparable antibacterial properties, their phenol coefficients

with respect to *S. typhosa* being 28.5 and 27.5, and with respect to *M. pyogenes* var. *aureus* 45 and 44. This close similarity in bactericidal efficacy is understandable on the grounds of chemical constitution, both compounds being isomeric methyl isopropyl phenols.

Chlorothymol makes a satisfactory antiseptic when applied in the form of a solution in alcohol and glycerine (Beck, 1933). It has a phenol coefficient of 106.6 (Woodward, Kingery and Williams, 1933-34). Mono- and dichloro derivatives of carvacrol were found to be particularly effective against *M. pyogenes* var. *aureus* (Kuhn, 1931) comparing favorably with the corresponding thymol derivatives. "Carvasept" is a combination of chlorocarvacrol with soap; its phenol coefficient is 45 (Hoder, 1931).

*p*-Chloro-*m*-cresol (3-methyl-4-chlorophenol) has gained considerable importance as a component of an industrial preservative, under the name of "Collatone." *p*-Chloro-*sym.m*-xylenol (3,5-dimethyl-4-chlorophenol) is used, among other things in Liquor Chloroxylenolis of the British Pharmacopoeia.

The germicidal action of 2,6-dichloro-4-*n*-alkyl phenol derivatives was studied by Blicke and Stockhaus (1933). 2,4-Dichloro-*m*-xylenol gained some prominence recently as an ingredient of "degerming" soap formulations (Gemmell, 1952).

The formulation of disinfectants with benzyl phenols and their halogen substitution products is the subject of a study by Carswell and Doubly (1936). A liquid soap properly formulated with *p*-chloro-*o*-benzyl phenol (5-chloro-2-hydroxydiphenyl methane) compared favorably with one containing the same proportion of Hexachlorophene in regard to "degerming" effectiveness (Bowers, 1950).

The ratio of soap to *p*-chloro-*o*-benzyl phenol is critical with regard to the bactericidal effectiveness of the combination. Bean and Berry (1951), working with *p*-chloro-*o*-benzyl phenol and potassium laurate, showed that the bactericidal activity of the solutions is related to the concentration of the benzylchlorophenol in the micelles of the potassium laurate, and independent of the overall concentration in the solutions. An increase in the proportion of benzylchlorophenol to potassium laurate produces a marked increase in bactericidal activity.

In the recent past several phenyl phenols and their monohalogenated derivatives gained considerable importance. Tilly, MacDonald and Schaffer (1931) reported *o*-phenyl phenol to be an effective tuberculocidal agent. *o*-Phenyl phenol combined with potassium ricinoleate furnishes the active ingredient of "O-syl." The same compound, plus the xylenol fraction of cresylic acid in combination with soap occurs in "Lysol"; with *p*-tert amyl phenol and potassium cinoleate it makes up the active principle of "Amphyl."

The following Table 84 illustrates the microbicidal efficacy of the saponated compound of *o*-phenyl phenol ("O-syl") in terms of phenol coefficients.

TABLE 84.—PHENOL COEFFICIENTS OF "O-SYL"

Microorganisms	20° C.	37° C.
<i>Salmonella typhosa</i>	5.6	—
<i>Salmonella paratyphi</i>	6.6	7.2
<i>Shigella paradysenteriae</i>	—	8.8
<i>Escherichia coli</i>	5.0	5.3
<i>Proteus vulgaris</i>	4.0	4.5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	3.3	4.0
<i>Streptococcus viridans</i>	—	6.2
<i>Streptococcus pyogenes</i> (hemol.)	—	5.6
<i>Neisseria catarrhalis</i>	—	5.5
<i>Diplococcus pneumoniae</i> , Type III	—	8.0

This type of phenolic disinfectant enjoys a great advantage over the products of the type of Liquor Cresolis Sap. (N.F. or U.S.P.) in that it offers substantial freedom from the characteristic disinfectant odor, coupled with practical absence of risk of toxicity or corrosiveness to tissue.

Compared to disinfectants of the character of Saponated Solution of Cresol, those formulated with phenolic compounds of low volatility have a noteworthy point in their favor, *viz.*, that of imparting an enduring antibacterial potential to disinfected surfaces (Klarmann, Wright and Shternov, 1953). This is in marked contrast, *e.g.*, to the comparatively fleeting effect of hypochlorites. Properly formulated phenolic disinfectants are substantially non-specific in regard to their bactericidal and fungicidal action, and thereby qualified for use in disinfecting practice which is directed logically against pathogenic vegetative microorganisms in general, rather than against any species or class in particular.

Incidentally, it has been observed (Klarmann, 1934, 1937) that combinations of certain alkyl and aryl phenols with other phenol derivatives or with terpineol may display a considerable potentiation of antibacterial power as compared with the theoretical effectiveness derived on an additive basis.

Conversion of phenol and of the phenol derivatives to the corresponding sodium or potassium phenates causes a reduction in bactericidal power; on the other hand, the phenates are much more soluble in water than the phenols from which they are derived. Moreover, in certain cases, the phenates may act as "solubilizing" agents for the free phenols. In other words, to effect solution of a phenolic compound by means of alkali it is not always necessary



to use the stoichiometrically required proportion of the latter; in some instances, *e.g.*, half the proportion needed for complete neutralization is sufficient to effect solution. The advantage obtained from this property is that, other things being equal, the resulting compound displays a greater microbicidal power than it would show in the case of complete neutralization.

Whether or not use may be made of this property depends upon the individual phenol derivative. In fact, some phenols require an excess of alkali over that calculated on a stoichiometrical basis. They still may be useful under certain conditions directly in the form of their solutions (with a high pH); or the alkaline solutions may serve as a means of depositing such phenols in the form of poorly soluble precipitates upon surfaces (fabrics, leather, paper board) etc. which are to be protected against attack by airborne microorganisms (bacteria, molds and yeasts). Often exposure to the carbon dioxide of the atmosphere suffices to liberate the phenol from its phenate combination with attendant fixation of the "insoluble" phenol upon the surface to which it has been applied as the soluble phenate.

To use an example, 2,4,5-trichlorophenol shows a phenol coefficient of 40 when tested at pH 6 against *S. typhosa*; at pH 10 or above, the phenol coefficient drops to about 1. To protect a given suitable material against microbial action, one could apply either the free trichlorophenol in a volatile solvent (*e.g.*, acetone) or as the corresponding sodium salt in aqueous solution. As a matter of fact, in spite of the wide divergence in the phenol coefficients, both forms furnish approximately equal protection against microbial spoilage to the material to be preserved.

2-Chloro-4-phenyl phenol gives a phenol coefficient against *S. typhosa* of 120 to 130, 2-bromo-4-phenyl phenol one of 140 to 160. The phenol coefficients of the corresponding sodium salts are respectively 73 to 80, and 128 to 147. It is quite evident that in these cases, unlike in that of 2,4,5-trichlorophenol, conversion into the sodium salts while causing a distinct reduction in bactericidal potency, did not bring about anything like an almost complete abolition of this potency.

Undoubtedly, this phenomenon depends upon the difference in the dissociation constants of the two phenol derivatives under consideration. It is known that the acidic characters of phenol derivatives is a function of the degree of their halogenation. Thus a trichloro derivative, being distinctly acidic in character and therefore possessed of a comparatively high dissociation constant, will yield an almost completely dissociated sodium salt. By contrast, a monochloro derivative representing a weak acid (with a low dissociation

constant) will yield a sodium compound with a marked tendency toward hydrolysis. Its aqueous solution will contain some of the free phenolic substance and, therefore, the apparent degree of impairment of the antibacterial effect will be less in this case than in that of a polyhalogen phenol derivative.

No noteworthy germicidal activity was encountered by Col. Prouty and Meserve (1941) among several substituted naphthol derivatives. Thus, 4-bromo-2-naphthol, 2-butyro-1-naphthol, 4-bromo-2-propionyl naphthol, 2-propyl-1-naphthol ethyl ether were practically ineffective against *S. typhosa*, *E. coli*, *B. subtilis* and *M. pyogenes* var. *aureus*; however, 2-ethyl-1-naphthol killed all test organisms in 30 minutes, and 2-propyl-1-naphthol all but *E. coli*.

It cannot be repeated often enough that the phenol coefficient alone (as obtained, *e.g.*, with *S. typhosa* as test organism) need not furnish an adequate criterion of the general disinfectant efficacy of a given phenolic compound. As pointed out by Brewer (1944), the phenol coefficient method is being used to an ever increasing degree for the testing of products for which it was not intended. Many new antibacterial agents are employed as replacements for cresylic and other coal-tar disinfectants for which the phenol coefficient method was devised originally. Brewer recognized several factors as being mainly responsible for the inaccuracies and inconsistencies observed in testing many of these newer germicides by the phenol coefficient method. At any rate, it is quite clear that in order to gain an idea as to a given disinfectant's capacity to perform under the particular conditions of its intended use it is necessary to introduce such modifying factors into the testing method as would make it reflect as nearly as possible the conditions encountered in practice. Thus, where one is concerned, *e.g.*, with the disinfection of comparatively clean surfaces, the use dilution of a disinfectant prepared on the basis of some unmodified laboratory procedure may be found adequate for the task. On the other hand, the disinfection of a surgical instrument contaminated with blood or exudate should not be entrusted to a solution whose antibacterial capacity has been determined in the absence of such or similar contaminating material.

The discussion of the important class of symmetrical polyhalogendihydroxydiphenyl methane derivatives including Hexachlorophene could be dealt with in this chapter. However, the reader is referred to a separate special chapter dealing with this subject, also with that of "Actamer."

## DIHYDRIC PHENOLS AND DERIVATIVES

An extensive study of the antibacterial properties of resorcinol derivatives began with the basic findings of Johnson and his co-

workers (Johnson and Hodge, 1913; Johnson and Lane, 1921). Additional data were contributed by investigators entering this field subsequently (Dohme, Cox and Miller, 1926; Schaffer and Tilley, 1926, 1927; Hampil, 1928; Rettger, Valley, and Plastring, 1929). Hampil has shown that in the homologous series of alkyl derivatives of resorcinol, temperature affects the position of the several homologs when rated according to their germicidal efficacy. Thus at

TABLE 85.—COMPARISON OF THE BACTERICIDAL ACTION OF RESORCINOL MONOETHERS WITH THAT OF THE CORRESPONDING NUCLEUS SUBSTITUTED RESORCINOL DERIVATIVES. (A, MONETHERS; B, O-P-NUCLEUS SUBSTITUTED DERIVATIVES.) PHENOL COEFFICIENTS, 37° C.

	<i>Salmonella typhosa</i>		<i>Micrococcus pyogenes</i> var. <i>aureus</i>	
	A	B	A	B
Resorcinol (VI)	0.4	0.4	0.4	0.4
Methyl VI	1.3		1.2	
Ethyl VI	3.6		3.0	1.5
n-Propyl VI	6.9	5.0	5.4	3.7
n-Butyl VI	20	22	18	10
n-Amyl VI	38	33	36	30
n-Hexyl VI	46	46-56	125	98
n-Heptyl VI	21	30	330	280
n-Octyl VI	2	0	580	680
n-Nonyl VI	3.4	0	650	980
Phenyl VI	40		37	
Benzyl VI	21	18	16	14
Phenylethyl VI	35	41	39	21
Phenylpropyl VI	34	31	89	
p-Chlorobenzyl VI	61	63	38	40

20°C heptyl resorcinol appears to be more effective than its nearest neighbors, viz., the hexyl and octyl derivatives, while at 37°C the octyl derivative is the most effective; at 45°C nonyl resorcinol displays the greatest efficacy although this compound shows practically no activity at 20° and little of it at 37°C.

Klarmann, Gates and Shternov (1931) prepared and studied the series of monoethers of resorcinol. The following Table 85 compares the bactericidal effects of the 4-alkyl resorcinols with those of the corresponding resorcinol monoethers (in terms of phenol coefficients). The data pertaining to the former class are those reported by Dohme, Cox and Miller for *S. typhosa*, and by Tilley and Schaffer for *M. pyogenes* var. *aureus*; this table contains also some comparative data bearing upon substitution of resorcinol by aromatic groups (Klarmann, 1926a).

The data given in Table 86 justify the conclusion that the bactericidal effectiveness of substituted resorcinol derivatives is practically the same regardless of whether the substituting radical



is in the nucleus in the ortho-para position to the two hydroxyl groups, or attached to one of the oxygen atoms. It follows also that one hydroxyl group of the resorcinol molecule is sufficient to bring about the antibacterial action, probably without the participation of the other hydroxyl group, if both are open (as in the case of the nucleus substituted resorcinol derivatives); it appears that the effect of the resorcinol monoethers (with one open hydroxyl group) is quite similar to that of the corresponding nucleus substituted derivatives (with both hydroxyl groups open).

TABLE 86.—COMPARISON OF THE BACTERICIDAL ACTION OF THE MONOETHERS OF THE THREE DIHYDRIC PHENOLS (PHENOL COEFFICIENTS, 37° C.) RESORCINOL (VI), HYDROQUINONE (VII), PYROCATECHOL (VIII).

Radicals	<i>Salmonella typhosa</i>			<i>Micrococcus pyogenes</i> var. <i>aureus</i>		
	VI	VII	VIII	VI	VII	VIII
None	0.4	>12	0.9	0.4	0.4	0.6
Methyl	1.3	1.0	0.9	1.2	0.8	0.7
Ethyl	3.6	1.5	1.8	3.0	1.5	1.6
n-Propyl	6.9	5.4	4.1	5.4	4.1	3.8
n-Butyl	20.0	14.0	9.8	18.0	9.3	10.0
n-Amyl	38.0	29.0	22.0	36.0	30.0	23.0
sec Amyl	26.0	19.0	18.0	31.0	26.0	20.0
n-Hexyl	46.0	18.0	17.0	125.0	100.0	28.0
n-Heptyl	21.0	17.0	9.7	330.0	200.0	37.0
n-Octyl	2.3			580.0	360.0	
Phenyl	40.0	41.0	17.0	37.0	28.0	11.0
Benzyl	21.0	21.0	19.0	16.0	14.0	15.0
Phenylethyl	35.0	25.0	22.0	39.0	29.0	21.0
Phenylpropyl	34.0	10.0	15.0	89.0	13.0	19.0

As in the case of the phenol derivatives so also in that of the resorcinol derivatives a "quasi-specific" effect is in evidence, in that with respect to the test-organism *M. pyogenes* var. *aureus* the bactericidal efficacy increases markedly with the increasing weight of the radical, whereas with respect to *S. typhosa* a maximum is reached by the *n*-hexyl derivatives, followed by a sharp decline; it is not sure whether the maximum of efficacy with respect to *M. pyogenes* var. *aureus* has been reached with the *n*-nonyl derivative which appears to be without any effect upon *S. typhosa*.

Klarmann, Gates, and Shternov (1932a) studied also the monoethers of the other two dihydric phenols, viz., hydroquinone and pyrocatechol. (Hydroquinone itself has an unusually high temperature coefficient of germicidal action upon *S. typhosa*, but not upon *M. pyogenes* var. *aureus*; by contrast, its monoethers do not display this effect.) Among the hydroquinone and pyrocatechol monoethers there are a number of effective antibacterial agents.

although in general, the monoethers of resorcinol appear to show a greater efficacy than those of the other two dihydric phenols.

Table 86 gives a comparison of the bactericidal action of the three monoethers, in terms of phenol coefficients (as determined at 37° C).

It appears from this table that the "quasi-specific" effect referred to before is found also in the case of the monoethers of hydroquinone and of pyrocatechol.

As in the case of resorcinol, so also in that of pyrocatechol, a comparison is possible between the monoalkyl ethers and the

TABLE 87.—COMPARISON OF ISOMERIC HYDROXYDIPHENYL OXIDES AND SULFIDES (PHENOL COEFFICIENTS, 37° C.).

	Oxide	Sulfide
<i>o</i> -Hydroxydiphenyl	17	33
<i>m</i> -Hydroxydiphenyl	40	68
<i>p</i> -Hydroxydiphenyl	41	115

corresponding nucleus substituted alkyl derivatives, since several of the latter were prepared and studied by Rawlins and Hamilton (1938). In contradistinction to the situation found in the case of the resorcinol derivatives, the 4-alkyl derivatives of pyrocatechol yield substantially higher phenol coefficients than their monoether isomers; in fact their antibacterial potency appears to compare favorably with that of the corresponding 4-*n*-alkyl resorcinols (and the analogous resorcinol monoethers).

The phenyl ethers of the three dihydric phenols which may be regarded also as isomeric hydroxyphenyl oxides, permit a comparison with the corresponding isomeric sulfides prepared by Hilbert and Johnson (1929). Table 87 compares the effects upon *S. typhosa* of these two isomeric series.

It appears that with respect to *S. typhosa* as test organism, the three hydroxydiphenyl sulfides are more effective than the corresponding oxides.

A number of symmetric dihydroxydiphenyl sulfides were prepared and studied by Dunning, Dunning, and Drake (1931). These sulfides were derived from the following initial compounds: phenol, resorcinol, *m*-cresol, *p*-chloro- and *p*-bromophenol and thymol. The anti-bacterial potency of the sulfides is several times that of the initial phenol derivatives.

Of the several nucleus substituted alkyl resorcinol derivatives studied, 4-*n*-hexyl resorcinol has acquired considerable practical significance. Although in the early stages of its investigation it seemed possible that the combination of high antibacterial potency

with low toxicity might yield a drug of chemotherapeutic utility, it was found soon that such a hope was not justified. Nevertheless 4-*n*-hexyl resorcinol became an important antiseptic for topical use. It was accepted into the U. S. Pharmacopoeia; a solution of it with low surface tension is employed widely as a popular antiseptic for first-aid and other uses.

According to Hurd and McNamee (1937), 4-hexenyl resorcinol resembles 4-hexylresorcinol in its germicidal power as shown by the phenol coefficients (determined at 37.5°C) of 150 with respect to *M. pyogenes* var. *aureus*, 200 to *S. pyogenes* and 40 (determined at 20.5°C) to *S. typhosa*. 4-*cyclo*Hexyl and 4-*isocyclo*Hexyl resorcinol are less active than the corresponding *n*-hexyl derivative (Bartlett and Garland, 1927); the same appears to be true of the isomeric *cyclopentyl* methyl resorcinol (Talbot and Adams, 1927).

Three aromatic nucleus substituted derivatives of resorcinol were prepared by Klarmann (1926 *a, b*). They are the 4-benzyl, 4-phenylethyl and 4-phenylpropyl derivatives (also to be identified as 2,4-dihydroxydiphenylmethane, -ethane and -propane). Their phenol coefficients are respectively 23, 41, and 31. According to Suter and Smith (1939), 4-phenyl resorcinol gives a phenol coefficient of 14 against *M. pyogenes* var. *aureus* at 37°C; 5-phenyl resorcinol gives one of less than 12 under the same conditions.

Suter and Weston (1939) prepared several 5-*n*-alkyl resorcinols. These were found to resemble the corresponding 4-*n*-alkyl derivatives in regard to their antibacterial action upon *M. pyogenes* var. *aureus*; however they were much weaker against *S. typhosa* than the latter. The 2-alkyl resorcinols (Russell, Frye, and Mauldin, 1940) are even less effective.

A group of 4,6-dialkyl resorcinols were prepared and studied by Klarmann (1926*b*). It was found that 4,6-diethyl resorcinol with a phenol coefficient of 10, corresponded in its antibacterial action to the isomeric 4-*n*-butyl resorcinol which gave a phenol coefficient of 8; however 4,6-dipropyl resorcinol, with a phenol coefficient of 18, was less effective than the corresponding 4-*n*-hexylresorcinol with a phenol coefficient of 45. Very high phenol coefficient figures have been claimed for several members of a series of di-*sec* alkyl resorcinol derivatives, with respect to *M. pyogenes* var. *aureus* and *S. pyogenes* test organisms (Miller, Hartung, Rock, and Crossley, 1938).

As in the case of the alkyl phenol derivatives, so also in that of the alkyl resorcinols, the introduction of chlorine into the nucleus results in a marked intensification of the antibacterial action, particularly against *M. pyogenes* var. *aureus*. The pertinent studies were carried out independently by Read, Reddish, and Burlingame



(1934), and by Moore, Day, and Suter (1934). Chlorination of resorcinol monoethers also brings about an enhancement of the antibacterial potency, but the effect of chlorination is less pronounced in this case than in that of nucleus substituted alkyl resorcinol derivatives, according to the former authors.

Halogenated benzyl resorcinol derivatives are the subject of an investigation by Klarmann and Von Wowern (1929). The enhancement by halogen substitution of the antibacterial action of 4-benzyl resorcinol (2,4-dihydroxydiphenyl methane) and the effect upon this enhancement of the position of the substituting halogen is illustrated by the following Table 88.

TABLE 88.—BACTERICIDAL ACTION OF CHLORO- AND BROMO- DERIVATIVES OF 4-BENZYL RESORCINOL (2,4-DIHYDROXYDIPHENYLMETHANE). (PHENOL CO-EFFICIENTS, 20° C.).

	<i>Salmonella</i> <i>typhosa</i>	<i>Micrococcus</i> <i>pyogenes</i> var. <i>aureus</i>
2,4-Dihydroxydiphenylmethane (IX)	18.3	11.3
5-Chloro- IX	48.2	36.7
4'-Chloro- IX	63.1	40.1
5-Bromo- IX	37.3	44.6
4'-Bromo- IX	54.8	51.4

In the presence of added organic matter, the performance of the higher alkyl resorcinols is depressed to a very marked degree. A similar effect is brought about by soaps, such as sodium oleate and myristate, or potassium palmitate and stearate (Hampil, 1928).

Esterification of hexyl resorcinol with sulfuric acid leads to a comparatively ineffective compound. Unlike the parent substance the sulfate is not capable of reducing the surface tension of water.

Dialkylaminoalkyl ethers of hexyl resorcinol (and of stilbestrol) are claimed to be effective against *M. tuberculosis*, less so against *S. typhosa* and *M. pyogenes* var. *aureus* (Chapman, Hager, and Shay, 1947).

## DERIVATIVES OF TRIHYDRIC PHENOLS

Phloroglucinol is not an effective germicide. According to Cooper (1912), its phenol coefficient with respect to *S. typhosa* is only 0.35. However, introduction of alkyl or aralkyl radicals into its nucleus brings about a marked potentiation of its antibacterial efficacy. Klarmann and Figdor (1926) prepared the *n*-hexyl derivative whose phenol coefficient of 8 is over 3 times that of the isomeric triethyl phloroglucinol with a phenol coefficient of 2.5. The *S. typhosa* phenol coefficients of the three aralkyl phloroglucinols tested,

*viz.*, of the benzyl, phenylethyl and phenylpropyl derivatives are also around 8 (Klarmann, 1926*b*).

Pyrogallol, too, is a comparatively weak bactericide, although somewhat more effective than phloroglucinol. Its *S. typhosa* phenol coefficient is 0.77. However the series of 4-*n*-alkyl derivatives of pyrogallol contains several comparatively potent bactericides (Hart and Woodruff, 1936); the "quasi-specific" phenomenon is in evidence in that with respect to *E. coli* the maximum effectiveness is reached by the 4-*n*-hexyl compound (phenol coefficient 38) whereas with respect to *M. pyogenes* var. *aureus* such maximum may not have been attained with the 4-*n*-heptyl derivative (phenol coefficient 50). The 4-*n*-heptenyl pyrogallol is credited with a phenol coefficient of 120 with respect to *M. pyogenes* var. *aureus* (Hurd and Parrish, 1935).

The third isomeric trihydric phenol is hydroxyhydroquinone. Neither this compound nor its derivatives appear to have been examined for any antibacterial properties.

## HYDROXYCARBOXYLIC ACIDS AND ESTERS

Ortho-hydroxybenzoic or salicylic acid has been the subject of several studies. The wide discrepancy of ideas as to its antibacterial value is exemplified by the findings of Caius and his coworkers (1927) on one hand, and those of Woodward and collaborators (1933) on the other; the former find salicylic acid less effective than phenol with respect to *Pasteurella pestis* whereas the latter attribute to it a phenol coefficient as high as 13.3. Mono- and di-chloro and -bromo derivatives of salicylic acid have been prepared and investigated for both their killing and inhibitory action upon bacteria, fungi and yeasts (Delauney, 1937; Rochaix and Pinet, 1927).

The phenol coefficient of *m*-hydroxybenzoic acid is around 2.7 with *S. typhosa*, and 2.1 with *M. pyogenes* var. *aureus* as test organisms (Wyss and Poe, 1945).

A position of considerable importance is occupied by the esters of *p*-hydroxybenzoic acid, notably in the field of preservation of carbohydrates, gums, proteins, and of other organic materials of industrial, pharmaceutical, and cosmetic significance against spoilage by air-borne microorganisms. The bulk of the original data has been contributed by Sabalitschka and his coworkers over a period of several years. (Sabalitschka, 1924*a*, 1924*b*, 1931, 1932; Sabalitschka and Liedege, 1934; Sabalitschka and Lietz, 1931). Gershenfeld and Perlstein (1939) regarded these esters as being among the most useful preservatives for pharmaceutical and related

preparations available at the time of the publication of their report. A tabulation indicating the proper concentration of the several esters required for preservation was prepared by Suess (1936).

Recently Sokol (1952) subjected the methyl, ethyl, propyl, and butyl *p*-hydroxybenzoates to an investigation of their antibacterial and antifungal properties, in an endeavor to resolve a number of contradictions in previous reports arising from the use of different test organisms and different testing methods. Table 89 indicates the concentration of these four esters required to effect complete inhibition of growth.

TABLE 89.—INHIBITION OF BACTERIAL AND FUNGAL GROWTH BY ESTERS OF *p*-HYDROXYBENZOIC ACID (PERCENTAGES).

Microörganism	Methyl	Ethyl	Propyl	Butyl
<i>Salmonella typhosa</i>	0.2	0.1	0.1	0.1
<i>Escherichia coli</i>	0.4	0.1	0.1	0.4
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	0.4	0.1	0.05	0.0125
<i>Proteus vulgaris</i>	0.2	0.1	0.05	0.05
<i>Pseudomonas aeruginosa</i>	0.4	0.4	0.8	0.8
<i>Aspergillus niger</i>	0.1	0.04	0.02	0.02
<i>Rhizopus nigricans</i>	0.05	0.025	0.0125	0.00625
<i>Chaetomium globosum</i>	0.05	0.025	0.00625	<0.003125
<i>Trichophyton interdigitale</i>	>0.008	0.008	0.004	0.002
<i>Candida albicans</i>	0.1	0.1	0.0125	0.0125
<i>Saccharomyces cerevisiae</i>	0.1	0.05	0.0125	0.00625

Esters other than the four listed in Table 89 were tested for their inhibitory action upon *Aspergillus niger*. Among them were the hexyl, cyclohexyl, *p*-chlorobenzyl, tetrahydrofurfuryl, glyceryl, and pentaerithrytyl derivatives. None was found to be more effective than the propyl and butyl *p*-hydroxybenzoates.

It is of considerable practical interest that the esters of *p*-hydroxybenzoic acid display a low order of toxicity, either acute (Schubel and Manger, 1929; Schubel, 1930) or subacute (Cremer, 1935). The average lethal doses for cats, dogs, and rabbits (in gram per kilogram of body weight) of the methyl, ethyl, and propyl esters are respectively 3, 5, and 6. (Benzoic acid which is used widely as a food preservative, yields a lethal dose of 2 thereby appearing to be more toxic than any of the three *p*-hydroxybenzoates tested.) When administered intraperitoneally the methyl ester appears to be only one-fourth as toxic as the free *p*-hydroxybenzoic acid. As to subacute toxicity tests, 2 to 20 milligrams per kilogram per day of the lower esters given to rabbits, guinea pigs and rats for a period of 120 days appeared to be entirely harmless. Three times this quantity given to rats for 30 days also failed to produce any ill effects. On the basis of supplementary studies in-



cluding those of irritation, absorption, and excretion carried out with both animals and humans Sokol (1952) concludes that the esters of *p*-hydroxybenzoic acid approximate the requirements of an "ideal" pharmaceutical preservative as formulated by Gershenfeld and Perlstein (1939).

For a very extensive review of literature on the esters of *p*-hydroxybenzoic acid the paper by Neidig and Burrell (1944) should be consulted.

### NITROPHENOLS

The introduction of the nitro-group into the nucleus of phenol and cresol enhances the antimicrobial action (Cooper, 1913; Glaser and Prüfer, 1923; Glaser and Wulwek, 1924; Ishiware, 1924). While the work of these authors credits the *m*-nitrophenol isomer with the highest potency (about three times that of phenol itself), Mazzetti (1928) found *p*-nitrophenol the most effective with respect to *S. typhosa*, and *o*-nitrophenol most active against *M. pyogenes* var. *aureus*. According to Beutner, Cohen, and Beutner (1941) *o*-nitrophenol is a comparatively active bactericide whereas Caius, Naidu, and Jang (1927) find it comparable to phenol with respect to *P. pestis*. Woodward, Kingery, and Williams (1933) compared the effect of the three nitrophenol isomers upon *Monilia tropicalis* and assigned the following phenol coefficients to them: to the ortho-isomer 6.6, to the meta-isomer 5.3 and to the para-isomer 5.1.

A comparison of the inhibitory effect upon fermentation produced by 4-hydroxybenzoic acid and 3-nitro-4-hydroxybenzoic acid, as well as by several esters of each, points to a greater activity of the latter series.

The antiseptic action of 2,4-dinitrophenol was found to be weaker than that of phenol with respect to *P. pestis* (Caius and coworkers, 1927).

A comparison of six dinitrophenol isomers in regard to their inhibitory capacity was carried out by Lecoq, Landrin, and Solomides (1949). Of these, the 2,5-dinitrophenol was found to be the most active isomer. Its action upon streptococci and tubercle bacilli is particularly pronounced, the respective inhibitory dilutions being 1:200,000 and 1:1,000,000. It is effective also against other microorganisms (such as *S. typhosa*, *E. coli*, *M. pyogenes* var. *aureus*, etc.), but in higher concentrations comparable to those in which the other isomers show an inhibitory effect.

Picric acid (2,4,6-trinitrophenol) is more active than either phenol or mononitrophenol against both *S. typhosa* and *M. pyogenes* var. *aureus* (Cooper, 1913). A phenol coefficient of 6 has been ascribed to it by Tidy (1915) while its activity against *P. pestis*

has been noted by Caius and coworkers (1927). Woodward, Kingery, and Williams (1933) found it to be moderately effective against *M. tropicalis* (when brought into solution with the aid of sodium carbonate); the phenol coefficient with respect to the latter micro-organism was determined at 5.3.

### AMINOPHENOLS

The effect upon *P. pestis* of *p*-aminophenol is greater than that of *o*-aminophenol (Caius, Naidu, and Jang, 1927) while 2,4-diaminophenol is more active than either, and more so than phenol.

The inhibitory action of a series of para-alkyl derivatives of *o*-aminophenol upon a number of organisms (*S. typhosa*, *M. pyogenes* var. *aureus*, *Ps. aeruginosa*) was studied by Barber and Haslewood (1944, 1945). No substantial enhancement of the bacteriostatic effect was observed over that of the unsubstituted *o*-aminophenol.

### NAPHTHOL AND DERIVATIVES

Of the two isomers, *viz.*, *alpha*- and *beta*-naphthol, the latter has been explored more thoroughly as an antibacterial agent because of its greater economy and stability. In disinfectant formulations it enjoyed greater importance in the past than it does today. Thalhimer and Palmer (1911) assign to *beta*-naphthol a phenol coefficient of 12.5. A mutual potentiation of the antibacterial effects of *beta*-naphthol and formaldehyde has been claimed by Frei and Krupski (1915).

Of the several polybromonaphthols studied, the tri- and tetrabromo derivatives are particularly effective against staphylococci and streptococci while their activity with respect to *E. coli* and *S. paratyphi* is very low (Bechhold, 1909).

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## 22

# CHLORINE AND CHLORINE COMPOUNDS

## INTRODUCTION AND HISTORICAL REVIEW

CHLORINE, although one of the most widely distributed of elements, is never found in the free state in nature. It exists essentially in combination with sodium, potassium, calcium and magnesium. This element was discovered by the Swedish chemist, Scheele, in 1774; however, he was unaware of its elemental nature and called it dephlogisticated muriatic acid. It was not until 1810 that Sir Humphrey Davy definitely proved chlorine to be an element and gave it the name which it now bears.

As early as 1785, the first effort to utilize chlorine commercially as a bleaching agent was done by making a solution of chlorine in water. This failed because of the destructive effect of chlorine on fibers. In 1789 Berthollet prepared a chlorinated potassium hydroxide solution and marketed this under the name of Eau of Javelle. It was about in 1794 that Henry first produced chloride of lime and in 1798 Charles Tennant erected a plant in Glasgow, Scotland, for the production of chloride of lime, which later became known as bleaching powder. It was during the first half of the nineteenth century that the disinfecting and deodorizing properties of chloride of lime were first recognized. In 1854, a report of the first Royal Sewage Commission of Great Britain referred to the use of chloride of lime as a deodorant in London sewage. Watt, in 1851, found that sodium hypochlorite could be prepared by electrolyzing a solution of sodium chloride. Koch, in 1881, reported on the bactericidal properties of hypochlorites and this was undoubtedly the first instance in which it was clearly pointed out that the object in the use of hypochlorites in disinfection was to destroy microorganisms. The

Committee on Disinfectants of the American Public Health Association, in 1886, reported very favorably on the use of hypochlorite solutions as disinfectants.

It is believed that the first to draw attention to the purifying properties of the hypochlorites in water was Trabue in 1894. In North America, the first commercial attempt to purify water by means of chloride of lime was made by Johnson in 1908. This installation was made in the City of Chicago at the Bubbly Creek Filter Plant. The effluent was chlorinated to a dosage of 1.5 ppm chlorine and the results obtained were reported as very satisfactory. During the next few years the use of hypochlorites in water purification, both alone and in conjunction with filtration, became very popular and in 1911 it was estimated that over 800,000,000 gallons per day were treated in this manner.

With the advent of elementary chlorine as a commercial product, this has replaced to a degree the use of hypochlorites as agents for use in water purification and sewage treatment.

Today chlorine is shipped as a compressed gas in liquid form in cylinders having capacities of 100 and 150 pounds, in larger containers holding 2,000 pounds and in single unit tank cars. Chloride of lime, known as chlorinated lime, although still available, has been almost entirely transplanted with commercial preparations of calcium hypochlorite containing 70 per cent available chlorine. Also present on the American market are a wide variety of proprietary sodium hypochlorite preparations sold as household bleaches and disinfectants. Organic compounds containing available chlorine have been developed and are used extensively as bactericidal agents.

## CHEMICAL CONSTITUTION

The term "available chlorine", as used in hypochlorite preparations, is a measurement of the oxidizing power expressed in terms of an equivalent quantity of chlorine. It originated from an old method of determining the strength of chlorinated lime. This was done by measuring the volume of chlorine released from acidified chlorinated lime and the volume of the gas was expressed as "per cent chlorine available".

One of the present methods for the determination of available chlorine is the iodide-starch-sodium thiosulphate method. In this method the following reaction with calcium hypochlorite takes place:



In this reaction, 1 molecule of calcium hypochlorite, which contains but 2 atoms of chlorine, liberates 4 atoms of iodine from



potassium iodide. Since each atom of iodine is considered equivalent to an atom of chlorine, the available chlorine measured in terms of the iodine liberated is twice the actual chlorine concentration in calcium hypochlorite. Thus,  $2I_2$  is equivalent to  $2Cl_2$  and  $Ca(OCl)_2$  is equivalent to  $2Cl_2$ .

The molecular weight of  $Ca(OCl)_2$  is 142.92 and of  $2Cl_2$  is 141.84, thus the ratio is 1.0007:1. A product which contains 50 per cent calcium hypochlorite has an equivalent of 49.65 per cent available chlorine. With sodium hypochlorite, which contains but one atom of chlorine, two atoms of iodine are liberated from potassium iodide. Thus,  $NaOCl$  is equivalent to  $Cl_2$ . The mol wt of  $NaOCl$  is 74.46 and of  $Cl_2$  is 70.92 and the ratio is 1.049:1. A product containing 10 per cent sodium hypochlorite has an equivalent of 9.52 per cent available chlorine. The concentration of the available chlorine content of chlorine solutions used for sanitizing farm dairy utensils, food handling and processing equipment and drinking and eating utensils may be readily determined by test sets using the iodide-starch-sodium thiosulphate method, the starch-iodide test papers and the o-tolidin method of the United States Public Health Service.

## THE MECHANISM OF CHLORINE DISINFECTION

Andrewes and Orton (1904) were among the first to suggest that the active germicidal agent of hypochlorites is free hypochlorous acid. The hydrolysis reaction is:



This hydrolysis is depressed with increasing alkalinity. Holwerda (1928) calculated the per cent hypochlorite present in the form of hypochlorous acid for various pH values. Table 90 gives these percentages:

TABLE 90

pH	Hypochlorite present as undissociated HOCl (per cent)
4.0	almost 100.0
5.0	99.6
6.0	95.8
7.0	69.7
8.0	18.7
9.0	2.2
10.0	0.2

The question has been often raised if either the unhydrolyzed hypochlorite or the  $OCl^-$  ion possesses any germicidal power. It is well known that alkaline solutions of both sodium and calcium hypo-

chlorites, which have only very small amounts of hypochlorous acid present, do possess definite germicidal properties. This suggests that the  $\text{OCl}^-$  ion may also be a factor. As the traces of hypochlorous acid are used in the germicidal process, the hydrolysis equilibrium will be altered and more of this acid will be formed and the reaction continued. With alkaline hypochlorites the disinfection is slow at the start and then increases. Since the  $\text{OCl}^-$  ion possesses a positive chlorine atom, it must be considered that the ion may have germicidal power.

The killing of bacteria by hypochlorites seems to exist in two phases (1) the penetration of the active germicidal principle into the cell and (2) the chemical combination of this ingredient with the protoplasm of the cell which is directly responsible for the death of the organism.

The mechanism of the destruction of microorganisms by hypochlorous acid has never been determined due to the size of the organisms and the difficulty of detecting any chemical and structural changes in bacteria. Chang (1944) studied the destruction of the cysts of *Endamoeba histolytica* by chlorine and inorganic and organic available chlorine carrying compounds. He reported that (1) there was direct penetration of chlorine into the cysts and the order of their activity is chlorine, hypochlorous acid, p-sulfondichloramino-benzoic acid, dichloramine, succinchlorimide, and monochloramine; (2) the greater the concentration of titratable chlorine and the longer the period of contact, the greater was the penetration of chlorine into the cysts; (3) the hypochlorite  $\text{OCl}^-$  ion was found to be non-cyst penetrating and non-cysticidal; (4) the action of chlorine and chloramine compounds on cysts was attributed to the active chlorine which may oxidize or chlorinate the proteins in the cell; and (5) the experimental evidence obtained indicated that the liberation of nascent oxygen by hypochlorous acid was unlikely to occur. Chang believes that when the chlorine is in contact with the protoplasm, the changes taking place probably render the cyst non-viable long before the formation of the chloramine has a chance to poison the cysts.

## METHODS FOR DETERMINING GERMICIDAL ACTIVITY

Many methods have been proposed and used for the determination of the germicidal activity of available chlorine carrying compounds. Such methods as the Rideal-Walker, Hygienic Laboratory and Food and Drug Administration have been proposed and used for this purpose. However, it is the general consensus that these methods are not applicable to the evaluation of the germicidal prop-

erties of these compounds. Johns (1934) developed his own technique known as the Johns' Glass Slide Method. This was done in an effort to simulate actual conditions under which products containing these compounds are used for the destruction of bacteria present on farm dairy utensils and dairy plant equipment. It has an advantage in that it approximates more readily the conditions under which bacteria need to be destroyed when they are present in a moist film of diluted milk upon a surface rather than being suspended in a solution. Also, this method enables one to employ periods of exposure similar to those employed when these products are used under practical conditions. In this test the organisms which have survived during a definite contact period can be easily counted with the result that the operator can determine the per cent kill in this time.

Weber and Black (1948) developed a laboratory procedure for evaluating the practical performance of germicides proposed for sanitizing food utensils. In this method they used contact periods of 30 to 300 seconds and a 100 per cent kill. Weber (1950) modified this technique in his study on the germicidal activity of chloramine-T. Two essential modifications were the alteration of time intervals and the killing time that is required to kill 99.9999 per cent of the exposed organisms.

## HYPOCHLORITES

Hypochlorites are available on the American market as powders containing calcium hypochlorite and sodium hypochlorite combined with hydrated trisodium phosphate and as liquids containing sodium hypochlorite. The preparations containing calcium hypochlorite are chlorinated lime, products containing 70 per cent available chlorine and others ranging from 15 to 50 per cent.

Freshly prepared chlorinated lime contains 30 to 35 per cent calcium hypochlorite. Because of the nature of its chemical composition, this product is unstable and under average storage condition loses approximately 1 per cent of its original calcium hypochlorite content per month. Moisture and heat increase this deterioration. Chlorinated lime has been largely supplanted by the 70 per cent products, although it still may be found in small packages on the shelves of many drug and grocery stores from whom it is purchased for use as a household deodorant and disinfectant. The 70 per cent calcium hypochlorites are used for the chlorination of small municipal water supplies, swimming pool waters and sewage effluents; for the preparation of 1 per cent sodium hypochlorite solutions used as a bleach by laundries, for the in-plant chlorination



of water supplies of food processing and handling plants and the sanitization of plant utensils and equipment. The products containing 15 to 50 per cent calcium hypochlorite are essentially used for sanitizing farm dairy utensils, dairy plant equipment and restaurant and tavern glasses and dishes.

The products containing sodium hypochlorite are essentially liquids ranging in concentrations from 1 to 15 per cent. The 1 per cent sodium hypochlorite solution is marketed for personal hygiene use and as a household disinfectant. Products containing in the range of 5 to 12 per cent are sold primarily as household bleaches and disinfectants and for use as sanitizing agents for dairy utensils and equipment. The 15 per cent solutions are used to prepare a 5 per cent solution which is distributed under many trade names as household bleaches and disinfectants and are also purchased by laundries and dairy plants for use diluted as a bleach and sanitizing agent. The hydrated trisodium phosphate-sodium hypochlorite product is used for sanitizing dairy utensils and equipment.

*The Effect of pH on Germicidal Activity.*—It is a well-known fact that increasing the alkalinity of solutions having available chlorine derived from a hypochlorite decreases the bactericidal property, while with increase in acidity, increase in killing time results. Rideal and Evans (1921) were the first to indicate the influence of pH when the authors showed that the addition of an alkali caused a marked depression of the oxidizing power of a hypochlorite. Wright (1926) demonstrated that an increase in alkali in the presence of organic matter (alanine) caused a more rapid and complete destruction of the hypochlorite as measured by iodometrical titration. However, no bactericidal data were submitted to show if the destroyed titer of available chlorine corresponded to the decrease in bactericidal activity.

Johns (1930) was one of the first to report on the germicidal efficiency of various commercial hypochlorite products. He noted in the case of a chlorinated trisodium phosphate product of rather high alkalinity that the killing time was greater for mixed organisms obtained from milk cans and for *S. lactis* than that of a commercial liquid hypochlorite. This slower germicidal action is apparently due to the higher pH value of solutions containing the same available chlorine content as those prepared from the less alkaline liquid hypochlorites.

Further work by Johns (1934) showed that the germicidal potency of hypochlorites is dependent upon both the concentration of available chlorine and pH of the solutions or upon the amount of hypochlorous acid formed, which is actually dependent upon

both of these factors. The influence of the pH, particularly in dilute solutions, is even greater than the per cent of available chlorine.

Levine and Charlton (1937) used as a test organism a spore forming organism isolated from a sample of spoiled ginger ale and named by the authors *Bacillus metiens*. The spore form was used in order to determine differences in killing times between hypochlorite solutions having various pH values and available chlorine concentration because of the greater killing time required to kill spores. Products containing calcium hypochlorite as the active germicidal ingredient were used in these studies. Using a 100 ppm available chlorine solution having a pH 10.4, the authors found that this solution had about the same killing time (70 minutes) as one containing 1,000 ppm available chlorine having a pH 11.3. This showed that the germicidal action of the more dilute hypochlorite solution was equal to that of one 10 times more concentrated but more alkaline and that the lower pH of the 100 ppm solution was the factor responsible, for there was apparently more than enough available chlorine present even in the 100 ppm solution to satisfy completely the chlorine demand of the numbers of bacteria present. In another experiment, the original pH of the 1,000 ppm solution was reduced to 7.3 by the addition of acid and the destruction of the spores was greater than 99.9 per cent in 20 seconds. The authors concluded that the concentration of the undissociated hypochlorous acid is probably the determining factor in the ratio of bacteria destruction by the hypochlorites.

The question of whether or not chlorine exhibits bacteriostasis or bactericidal properties was raised by the work of Mudge and Smith (1935). These workers reported that plate counts showed as high as a 99 per cent reduction while serial dilution tubes showed no reduction from the original bacterial population when the organisms used were exposed to dilutions of a highly alkaline hypochlorite. They attributed their results to bacteriostasis and not death. Costigan (1937) compared the efficiency of high and low alkaline hypochlorites using the plate colony count and dilution tube methods and *Salmonella typhosa* and *Micrococcus pyogenes* var. *aureus* in the presence of 1 per cent sterile whole milk. Solutions containing 50, 100, and 200 ppm available chlorine prepared from each hypochlorite and exposure times of 3, 5, and 10 minutes were employed. Using the highly alkaline hypochlorite and *S. typhosa*, the author reports a 99.9 per cent reduction in both dilution tubes and plate counts and with *M. pyogenes* var. *aureus* the dilution tubes and plate counts were in agreement although the per cent reduction was not as great as with *S. typhosa*. With the low alkaline hypochlorite and these organisms, the dilution tubes and plate counts

were in agreement and showed a greater bacterial reduction in the same concentrations and times of exposure than the highly alkaline product. With both hypochlorites, the gram-negative organism, *S. typhosa*, showed less resistance than the gram-positive, *M. pyogenes* var. *aureus*. The author concluded that the hypochlorites are definitely bactericidal.

Levine and Rudolph (1941) reported on the effect of pH on the germicidal efficiency of a calcium hypochlorite using the spores of *B. metiens* and a killing percentage of 99. At pH 10 and temperature 20°C, the killing times obtained were 121 minutes for 25 ppm, 63.5 minutes for 100 ppm and 31 minutes for 500 ppm available chlorine. This shows that in general quadrupling the concentration reduced the killing time approximately 50 per cent, when comparing solutions of 25 and 100 ppm. Using 25 ppm, the authors found the killing time at pH 6 to be 2½ minutes and at pH 8 5 minutes. In this range, the effect of the change on the killing time was relatively slight. However, the effect of the change in reaction in the range pH 8 to pH 10 was significant, the killing times being 5 and 121 minutes respectively. A hundredfold increase in the range pH 6 to 8 doubled the killing time, whereas a similar increase in the range pH 8 to 10 increased the killing time 20-fold. The authors conclude that the changes in killing time appear to be associated with changes in concentrations of undissociated hypochlorous acid; in the range pH 6 to 8, this is small as compared with that in the range pH 8 to 10. This suggested to the authors that the concentration of undissociated hypochlorous acid is closely associated with the rate of kill by hypochlorite solutions. They have theorized that two phases exist in the killing of bacteria with hypochlorites; (1) the penetration of the active principle into the cell and (2) the chemical union of this principle with the protoplasm which is directly responsible for the death of the organism.

Johns and Chaplin (1949) using a modification of Johns' Glass Slide Technique, in which dried and moist 10 per cent milk films were treated with hypochlorite solutions at pH 7.2 and 10.5 containing 200 ppm available chlorine, found that solutions of low pH gave a 99.9 per cent kill in moist 10 per cent milk film in 4 seconds while those of the higher pH value required 8 seconds to give the same kill with the test organism, *M. pyogenes* var. *aureus*. With similar dried films 10 and 20 seconds respectively were required to obtain the same kill. It appears that the degree of hydration affects the speed of kill without altering the pH effect. The authors point out that the milk content of these films is much greater than would be encountered in practice of sanitizing dairy utensils and dairy plant equipment even after the most casual rinsing of equipment.



*Effect of Temperature.*—Levine and Rudolph (1941) studied the effect of temperature upon the killing time of a calcium hypochlorite using *B. metiens* spores at concentrations of 25 ppm, having a pH 10 and temperatures of 20°, 30°, 35° and 50°C. The killing times obtained were 121 minutes at 20°C, 65 minutes at 30°C, 38.7 minutes at 35°C, and 9.3 minutes at 50°C. From these data the authors calculated that in the range of 20° to 50°C, the killing times are reduced 46 to 66 per cent for each 10°C rise, and since the lag in germicidal action is lessened as the temperature increases, it is believed that there is an increased rate of diffusion of the germicidal agent into the cell.

It often has been said that hypochlorites should only be used in cool water as sanitizing agents, because in water of higher temperatures, they lose available chlorine. Solutions containing 50, 100 and 200 ppm available chlorine prepared from a proprietary sodium hypochlorite alkaline with calcium hydrate were kept at 55°C for 180 minutes. At the start and at intervals of 30, 60, 120, and 180 minutes, available chlorine determinations were made. Each solution showed at the end of the 180-minute period the same concentration as at the start. Since solutions prepared from hypochlorite products show an increase in germicidal activity with increase in temperature and no loss in available chlorine, their use as sanitizing agents for food handling and processing equipment need not be restricted to tap water temperature but may be advantageously employed at moderately high temperatures.

*Effect of Organic Matter.*—The effect of milk residues remaining on the surface of food handling and processing utensils and equipment upon the bactericidal properties of hypochlorites was considered early in the study of the use of this chemical as a sanitizing rinse.

Prucha (1927) was one of the first experimenters to report work on the effect of milk on these solutions and found that rapid losses developed when 1 and 5 per cent skim milk are added to chlorine solutions. Loveless (1934) studied the rate of loss of available chlorine in presence of 0.1 per cent whole milk using commercial hypochlorite products in concentrations of approximately 200 ppm available chlorine at temperatures of 70°, 120°, 160°, and 212°F during a period of 60 minutes. He reported that all the preparations showed some loss of available chlorine at 70°F and the rate of loss increased as the temperatures increased. Control experiments without milk showed no loss in available chlorine at any of the temperatures for 1 hour with the exception of a small loss in the solution prepared from the liquid sodium hypochlorite at 212°F.

Johns (1948) using a modification of his technique, *Escherichia coli* and *M. pyogenes* var. *aureus*, used fresh sanitizing solutions prepared from a liquid sodium hypochlorite and reported that his tests failed to show the expected germicidal reduction in the presence of added skim or whole milk. This is of particular significance when hypochlorites might pick up small amounts of milk when used as sanitizing agents for dairy utensils and equipment. However, it by no means indicates that all utensils and equipment should not be thoroughly cleaned free of all milk residues before sanitizing with hypochlorite solutions.

### DAKIN'S SOLUTION

Dakin's Solution is a solution of sodium hypochlorite containing between 0.45 and 0.50 per cent. It derived its name from its originator, H. D. Dakin (1915). The original solution was prepared using chlorinated lime, sodium carbonate and boric acid. The resulting solution contained sodium hypochlorite and boric acid. Daufresne (1916) attributed the irritating properties of this solution to the presence of boric acid and modified its composition by using sodium carbonate—bicarbonate in place of sodium carbonate and boric acid. This solution was found to be more stable and less irritating than the original. Dakin's Solution was used extensively during World War I for the irrigation of gunshot wounds by the Carrel-Dakin technique. At that time extensive studies were made and published on the solvent action, irritant properties, rate of reaction upon necrotic tissue and toxicity. Taylor and Austin (1918a) showed that this solution has marked solvent action on leukocytes, erythrocytes and plasma clots, but none on blood clots. Taylor and Austin (1918b) found that the rate of loss of the available chlorine is more rapid in contact with necrotic tissue than in contact with normal tissue. Taylor and Austin (1918c), in determining the toxicity of Dakin's Solution, employed white mice and guinea pigs. Increasing dosages were injected intraperitoneally into mice and subcutaneously and intraperitoneally into guinea pigs. The lethal dose of Dakin's Solution for white mice per 100 g body weight is 24 mgs sodium hypochlorite and intraperitoneally in 6.3 mgs sodium hypochlorite per 100 g body weight. Cullen and Taylor (1918) employed rabbits' ears for testing the irritant properties of this solution. They concluded that Dakin's Solutions, in which the pH range of 9.3 to 10.2 is maintained by the use of buffer salts, have practically the same degree of irritant action. Solutions which have pH value less than 9.3 and greater than 10.2 are intensely irritating.

## CHLORAMINES

The simplest of the chloramines is  $\text{NH}_2\text{Cl}$ , a chemical compound in which one of the hydrogen atoms of ammonia has been replaced by chlorine. This compound was discovered by Raschig (1907). The germicidal property of this compound was first noted by Rideal (1910). He observed that in the chlorination of sewage, the first rapid consumption of chlorine was succeeded by a slower action which continued for days, and this was accompanied by germicidal action after the free chlorine or hypochlorite has disappeared. He stated that it was evident that the chlorine was acting by substitution for hydrogen in ammonia and organic compounds yielding products which had germicidal activity. Race (1918) utilized this knowledge by adding ammonia to a bleach solution and because of the high cost of bleach when imported from Europe, was led to try out the process on a practical scale for the purification of water. After considerable investigation, Race showed that the ammonia and bleach must be mixed as dilute solutions avoiding prolonged contact. This proved to be successful in the purification of Ottawa, Canada water. It was determined by him that the most efficient proportions of available chlorine and ammonia are two parts by weight of the former to one part by weight of the latter. Race pointed out that real advantage in the application of the chlorine-ammonia process to water supply was the elimination of the after growth problem.

## CHLORAMINE-T

Dakin, Cohen, and Kenyon (1916) studied the preparation of other chloramine compounds and came to the conclusion that the compound, chloramine-T, was an excellent germicide and this compound was used quite extensively in the treatment of infected wounds during World War I. This compound has the chemical name sodium p-toluene sulfonchloramide. In this compound, the chlorine is available for germicidal purposes and has the linkage :NCl. It yields 25 per cent available chlorine and is quite stable. It is formed by the reaction between p-toluene sulfonamide and sodium hypochlorite.

Chloramine-T is a white, crystalline powder having a slight chlorous odor. It is freely soluble in water, a saturated solution at room temperature containing about 15 per cent. It melts at  $160^\circ$  to  $175^\circ\text{C}$ . Solutions of chloramine-T are stable, neither moderate exposure to heat nor light causing appreciable decomposition. Taylor and Austin (1918*a*) reported that chloramine-T does not



have the power of dissolving necrotic tissue, pus and plasma clots. Cullen and Taylor (1918) stated that a 2 per cent solution of chloramine-T has no irritant action. It is their opinion that this is due to the fact that the chlorine is released much more slowly in the chloramines than in the hypochlorites and that the irritating effects must be due to the readily available chlorine. Taylor and Austin (1918c) report that chloramine-T solutions are more toxic than Dakin's solution.

*Germicidal Action.*—There is considerable conflict among authors as to the germicidal properties of chloramine-T and the hypochlorites. For instance, Tilley (1920) concluded that on a weight basis, chloramine-T was less germicidal than hypochlorites but on per cent available chlorine, it was slightly more germicidal. In contrast, Myers (1930), Besemann (1928) and Holwerda (1930) report on the slow germicidal action of this compound. Much of this earlier work was done using the phenol coefficient method of test, which is not applicable to determining the germicidal properties of available chlorine carrying compounds. In this method of testing bactericidal effectiveness, the test culture is grown in a broth containing peptone and beef extract and these, when added along with the organisms to the medication tubes, are attacked rapidly by the hypochlorite and to a much lesser degree by the chloramine-T. The amount of available chlorine would not be constant during the entire time of exposure of the organisms.

The mechanism of the germicidal action of the chloramines has been the subject of much controversy in the literature. Rideal (1910) reported a slow germicidal action in sewage after the disappearance of free chlorine and believed that chlorine would substitute for hydrogen in the amino group of nitrogenous products present in sewage. Dakin and co-workers (1917) expressed the view that sulfonchloramides act as chlorinating agents only when the chlorine from the chloramine has an opportunity to attach itself to a second compound in which the nitrogen atom is united to less acid groups than those in the original chloramine. It is Johns' opinion (1934) that chloramine-T hydrolyzes to yield hypochlorous acid, which is responsible for its germicidal action as is believed to be the case with hypochlorites. Holwerda (1928) was unable to detect any hypochlorous acid present in a chloramine solution containing 10 ppm available chlorine. Schmelkes and Horning (1935) in their work on azochloramid state that there is no apparent direct relationship between the oxidation-potential and germicidal power of this chloramine.

Levine and Charlton (1937) reported on the germicidal effectiveness of chloramine-T using bacterial spores, *B. metiens*, and,

because at a concentration of 200 ppm available chlorine the death rate was very slow, used a concentration range of from 1,000 to 4,000 ppm available chlorine. These concentrations were used at pH 6.0 to 8.8 at 25°C and the alterations were made by acidification with dilute hydrochloric acid. In this work a reduction of 99 per cent in the numbers of viable spores was designated the "killing time." Using 1,000 ppm available chlorine solutions, the authors report killing times range from 97 to 15 hours for solutions from pH 8.6 to 6.0. Using 2,000 ppm available chlorine solutions, the killing time ranged from 64 to 5.4 hours for solutions pH 8.7 to 6.0. With 4,000 ppm available chlorine solutions, the killing times for solutions ranging from pH 8.8 to 6.0 were 23.5 to 2.6 hours. The effect of doubling the concentration of available chlorine at a pH 6.0 and 25°C is apparent. For example, at pH 6.0, the killing times are 15, 5.4 and 2.6 hours for the 1,000, 2,000 and 4,000 ppm available chlorine solutions respectively. Using a 2,000 ppm available chlorine solution at pH 8.7 and at 25°, 35°, 45° and 55°C, they found killing times of 64, 17, 5.5 and 1.55 hours respectively, while at pH 6.0 and at 25°, 35°, 45° and 55°C, the authors reported killing times of 5.4, 0.88, 0.16 and 0.03 hours respectively. The increases in temperature 10°C (in the range 25° to 55°C) resulted in a reduction of the killing time by about 71 per cent when using solutions having an initial pH 8.7. With solution pH 6.0, in the same temperature range, the killing time reduction is about 82 per cent.

Johns (1930) studied the speed of the germicidal action of preparations containing chloramine-T upon bacteria commonly occurring in milk. In this study he used organisms which were found in milk cans, *Streptococcus lactis*, *E. coli*, *Aerobacter aerogenes* and the spores of *Bacillus subtilis*. Also, a mixed culture of organisms obtained from milk cans was used. Using *S. lactis*, he reported that the chloramine-T products showed slow germicidal activity; in fact, the reduction in number of organisms did not occur until a period of contact of 60 seconds was used. Using *E. coli* as a test organism, he reports that the chloramine-T products showed slow germicidal activity as did the results using *A. aerogenes* and mixed culture from milk cans. Against the spores of *B. subtilis* again the chloramine-T products showed a very slow germicidal activity. In these bactericidal tests, the concentrations of the chloramine-T solutions ranged from 100 to 200 ppm available chlorine. Johns concluded that products containing chloramine-T were not suitable for use as an instantaneous rinse for farm dairy utensils.

Prucha (1927) reported that 170 ppm available chlorine from chloramine-T compounds gave good results in his practical tests

and that equally good results were secured when only 50 ppm of hypochlorite chlorine was used.

Myers and Johnson (1932) and Myers (1930) secured similar results as Johns reported and advised that greater concentrations and longer contact periods were necessary when chloramine-T products were used as compared with hypochlorites.

The Public Health Service Milk Ordinance and Code (1939) and the Ordinance and Code for Restaurants (1948) in lieu of hot water permit the use of hypochlorite compounds in a minimum concentration of 50 ppm available chlorine with an exposure of 2 minutes. Chloramine-T is permitted to be used in a similar manner and the recommendation is made that the solution be bactericidally equivalent to 50 ppm available chlorine as hypochlorite.

Weber (1950) reported on work to determine the concentration of available chlorine as chloramine-T which is bactericidally equivalent to 50 ppm available chlorine as hypochlorite. In these studies the procedure used was a modification of the Weber and Black Method in which *M. pyogenes* var. *aureus* as the test organism and the killing time to destroy 99.9999 per cent of the bacteria were used. The killing time at 25°C of chloramine-T solutions prepared from three chloramine-T commercial products at concentrations of 50, 250, 500, 1,000 and 1,500 ppm available chlorine at various pH levels was determined. With compound A at 50 ppm (pH 6.4), the killing time was 2.15 minutes and with 250 ppm (pH 6.9), the killing time was shortened to 0.97 minutes. With an increase, concentrations to 500 (pH 7.2), 1,000 (pH 7.5) and 1,500 ppm (pH 8.1), the killing time was increased to 1.26, 1.6 and 2.7 minutes respectively. This compound, as indicated, is poorly buffered and the gradual increase in pH with increase in concentration was sufficient to have an increased effect on killing time. Using compound B at 50 ppm (pH 8.3), 250 ppm (pH 8.3), 500 ppm (pH 8.3), 1,000 ppm (pH 8.2) and 1,500 ppm (pH 8.1), the killing times were 31.5, 15.5, 8.5, 4.4, and 2.4 minutes respectively. This compound is well buffered and showed a gradual reduction in killing time as the concentration increased. Using compound C at 50 ppm (pH 10.5), 250 ppm (pH 11.3), 500 ppm (pH 11.5), 1,000 ppm (pH 11.7) and 1,500 ppm (pH 11.9), the killing times were 372, 258, 141, 66, and 52 minutes respectively. This compound acted somewhat like compound B but is not so well buffered and more alkaline with the result of greater killing time at each ppm studied.

From the results reported by Weber, it is recognized, as in the case of hypochlorites, that pH and concentration must be considered in establishing the bactericidal uses of chloramine-T solution. In general, it may be stated that longer exposure periods for chlora-



mine-T solutions are required to sanitize food handling and processing utensils and equipment than are required for hypochlorite solutions. Weber concludes: "Concentrations of chloramine-T of at least 250 ppm at a reaction not greater than about pH 7.0 or 500 to 1,000 ppm at a reaction not more alkaline than pH 7.5 would appear to be as rapid in germicidal action (in the absence of organic matter) as 50 ppm of the slower (alkaline) hypochlorites."

### DICHLORAMINE-T

Dichloramine-T is p-toluene sulfondichloramide in which both hydrogens of the amino group of p-toluene sulfonamide are replaced by chlorine. It may be prepared by dissolving p-toluene sulfonamide in a solution of calcium hypochlorite and acidifying with acetic acid. Dichloramine-T is a pale yellow crystalline powder having a strong chlorous odor. It melts at 78° to 83°C. It is insoluble in water but soluble in chloroform and benzene. Chlorinated oils are solvents for this compound and in its application in medicine, chlorinated paraffine oil is employed as the vehicle. The use of this product, because of its insolubility in water, has been confined to use as an antiseptic in medicine.

### HALAZONE

Halazone is the name which has been given to p-sulfondichloraminobenzoic acid. The compound is prepared by chlorinating p-sulfonaminobenzoic acid dissolved in sodium hydroxide solution. It is a white powder having a strong chlorous odor and melts at 213°C. It is sparingly soluble in water. The solubility of the chemical in water is increased by the use of sodium chloride with anhydrous sodium carbonate and borax, which are used in the preparation of tablets for water disinfection. Dakin and Dunham (1917) first reported on its preparation and use in tablet form with the above chemicals for the disinfection of polluted water. The tablets prepared by the method of these authors contain 4 mg of Halazone and are suitable for the disinfection of a liter of water. The authors report that Halazone tablets prepared from thoroughly dry sodium chloride with either borax or sodium carbonate and stored in amber glass bottles will maintain their germicidal efficiency at temperature to 32°C for 5 months and would be useful for more than a year. Continuous exposure to 40° to 50°C reduced their efficiency by about 50 per cent in 3 months. Halazone in doses 25 to 50 times that required for water disinfection appears to be non-toxic and this product is probably converted quantitatively in

the animal body to p-sulfonamidobenzoic acid. These authors report that this product is an excellent bactericide killing *E. coli* in tap water (New York City, Croton Reservoir) at concentration 1:500,000 in 30 minutes and in tap water plus 5 per cent sewage at concentration 1:330,000 in 60 minutes. Using *S. typhosa*, *Salmonella paratyphi A*, and *V. comma* in tap water, it was found that concentrations of 1:225,000, 1:225,000 and 1:450,000 respectively killed in 20 minutes. In tap water plus 5 per cent sewage, results show that a concentration 1:333,333 killed *S. typhosa* and *S. paratyphi A* in 40 minutes and *V. comma* in 20 minutes.

### DICHLORODIMETHYL HYDANTOIN

Dichlorodimethyl hydantoin is an organic available chlorine carrying compound. This chemical is used as the active bactericidal ingredient of a commercial sanitizing product used in the food industry. This product contains 25 per cent dichlorodimethyl hydantoin, which is equivalent to 16 per cent available chlorine. Johns (1951) compared the bactericidal properties of this product with calcium and sodium hypochlorite commercial products. In this work, he used the Johns' Glass Slide and Weber and Black Methods, the organisms *E. coli*, *M. pyogenes* var. *aureus* and *Pseudomonas aeruginosa*, exposure periods ranging from 21½ to 300 seconds and concentrations of 10 to 50 ppm available chlorine. Also, short exposure periods and low concentrations were used in order to determine if a difference exists in the time necessary to reduce the numbers 99.9 and 99.9999 per cent.

It was found that the difference in the rate of kill between the products studied was most noticeable using *E. coli*. Each hypochlorite product in the available chlorine concentration used and in short exposure times gave faster kill than the equivalent available chlorine concentration from dichlorodimethyl hydantoin. Using *M. pyogenes* var. *aureus*, Johns reported that this bactericide is somewhat more effective than the two calcium hypochlorites using solutions containing 25 and 50 ppm available chlorine. With *Ps. aeruginosa* it was shown that this chemical is slightly slower than the hypochlorites in its kill.

The pH values of 25, 50 and 100 ppm available chlorine solutions of the commercial product containing this bactericide in distilled water are 4.85, 4.63 and 4.43 respectively while that of all the hypochlorite products in strength of 100 ppm available chlorine in distilled water had values slightly above 10.

## SUCCINCHLORIMIDE

Succinchlorimide is the chlorinated imide of succinic acid. It is a white to yellowish-white powder having the odor of chlorine. It is sparingly soluble in benzene and chloroform and slightly soluble in ether. Its solubility in water is about 1.6 g per 100 ml at 25°C. Because of its slight solubility in water, it was necessary to devise a special formulation which made it quickly soluble when added to water at various temperatures. A 2-grain tablet containing 10 per cent succinchlorimide was prepared and used in the bactericidal studies.

Reddish and Pauley (1945) reported on this work using tap water and polluted waters of high chlorine demand at temperatures of 36°, 73° and 80°F. The organisms used were *S. typhosa*, *Shigella dysenteriae*, and *E. coli*. In heavily contaminated tap water at 73°F, one 2-grain tablet per quart of water killed all *S. typhosa* (304,000,000) in 10 minutes, all *S. dysenteriae* (117,400,000) within 5 minutes and all *E. coli* (100,000,000) within 10 minutes. In heavily contaminated river water at 36°F, one 2-grain tablet per quart of water killed all *S. typhosa* (75,600,000) within 10 minutes, all *S. dysenteriae* (28,800,000) within 5 minutes and all *E. coli* (33,300,000) within 10 minutes. In swamp water (15 ppm chlorine demand) at 80°F, one 2-grain tablet per quart of water killed all *S. typhosa* (288,000,000) within 5 minutes, all *S. dysenteriae* (828,000,000) within 5 minutes, and all *E. coli* (963,000,000) within 5 minutes.

Chang (1942) and Chang and Fair (1941) described a method of determining the cysticidal activity of this compound. Using tap water at 77°F, the authors report that six 2-grain tablets per quart of water killed 28,500 dysentery ameba cysts (*E. histolytica*) within 10 minutes and five 2-grain tablets killed the same number of cysts within 20 minutes.

In a careful study of the work of Stohlman and Smith (1944), Reddish and Pauley (1945) conclude that succinchlorimide is non-toxic in the cysticidal concentration used in decontaminating water, that this bactericide taken orally is non-toxic in a concentration even 23 times greater than the cysticidal dose and that taken orally it is toxic when the dosage is 320 times greater than the cysticidal dose.

SANITIZING AGENT FOR FOOD HANDLING AND  
PROCESSING EQUIPMENT

Available chlorine carrying solutions used for sanitizing food handling and processing equipment are prepared from both inor-



ganic, calcium and sodium hypochlorites, and organic, chloramine-T and dichlorodimethyl hydantoin containing products. Probably the first reference to this use was that of Whittaker and Mohler (1912) in which calcium hypochlorite was used as a sanitizing agent for milk bottles. The authors reported that immersion of bottles in a bleach solution of 3 parts per 100,000 parts of water reduced the bacteria count per bottle from an average of 120,000 to an average of 45. Since this initial article much has appeared in the literature on this use and no attempt will be made to review all the literature leading to the general acceptance of the use of chlorine for sanitizing food handling and processing equipment.

Prucha (1929) was one of the first of many agricultural station experimenters to study and report on the use of the available chlorine carrying compounds as agents for sanitizing farm dairy and milk plant equipment. The author in this bulletin gave his recommendations for the use of sanitizing solutions prepared from chlorinated lime, a proprietary liquid sodium hypochlorite, a chlorinated trisodium phosphate and preparations containing chloramine-T. He recommended (1) 50 to 100 ppm available chlorine for rinsing or pumping through large equipment, (2) 70 to 100 ppm for dipping utensils and (3) 200 ppm for spraying surfaces of large equipment. Prucha was also one of the first workers to advise that (1) these solutions be applied to utensils just before use, (2) the solution must come in contact with all surfaces long enough for the chlorine to kill the bacteria, usually 10 seconds or longer, (3) solutions effective only on utensils and equipment that are free from all soil and (4) these products should not be added to milk to attempt to control development of bacteria.

Loveless (1934) reported on the products containing calcium hypochlorite, proprietary liquid sodium hypochlorites and chlorinated trisodium phosphate, and products containing chloramine-T. This author, in addition to laboratory work, conducted studies on the practical application of chlorine bactericides on dairy farms. The commercial products used were a liquid sodium hypochlorite, a chlorinated trisodium phosphate, a calcium hypochlorite, a calcium hypochlorite with sodium carbonate, and a chloramine-T containing bactericide.

Stability studies on these proprietary products at room temperatures and in total darkness and diffused light during 12 months show the rate of deterioration to be in the descending order of liquid sodium hypochlorite, calcium hypochlorite, calcium hypochlorite with sodium carbonate, chlorinated trisodium phosphate, and chloramine-T products. The author reported that solutions of these products containing 200 ppm available chlorine when tested at

80° to 160°F for 60 minutes showed little or no loss in available chlorine content and concluded that "it seems safe to say that the effect of temperature alone on the stability of available chlorine in diluted solutions is practically nil."

In the presence of 0.1 per cent whole milk, it was found that all preparations used in solutions containing 200 ppm available chlorine with the exception of the one containing chloramine-T showed some loss at 70°F and increasing loss as temperature increased. The laboratory germicidal studies using *E. coli* showed that the products containing chloramine-T did not appear to be as efficient as the hypochlorites of equal available chlorine strength when acidified or tested at 160°F. The author's experimental work on 20 Vermont farms where the utensils were rinsed with the hypochlorite solution twice each day just before milking showed that the quality of the milk as measured by plate counts and methylene blue reduction tests was materially improved. Also, he concluded that the danger of off-flavors caused by chlorine solutions remaining on the interior surfaces of farm utensils appears to be negligible providing the solutions are carefully used and utensils drained.

Elliker, *et al.*, (1950) and Barber (1950) reported that on dairy farms satisfactory results were obtained when the farmer used the conventional washing method for utensils and milking machines and hypochlorite sanitizing just before use.

The United States Public Health Service Milk Ordinance and Code (1939) recommends chlorine as one of the agents for the bactericidal treatment of utensils between each usage. Item 14r of the Ordinance is deemed satisfied if all milk containers, utensils, strainer cloths and other equipment have been immersed in or exposed to a flow of a chlorine solution of approved strength for at least 2 minutes. The code states that there are several bactericides which may be used in complying with this ordinance. These consist generally of calcium hypochlorite, sodium hypochlorite or certain chloramine solutions. The code specifies that when calcium or sodium hypochlorite solutions are used as bactericidal rinses on dairy farms and at milk plants, these solutions must be discarded when the strength is reduced to 50 ppm available chlorine. When used as bactericidal sprays, the initial concentration must be of sufficient strength so that the solution which runs off or collects in the equipment contains at least 50 ppm. Solutions prepared from products containing chloramine or chloramine-T have slower bactericidal action than hypochlorites containing equal concentrations of available chlorine. These must be made up to a greater concentration in order to produce the bactericidal effect within the exposure period equivalent to the recommended hypochlorite strength.

This ordinance (Public Health Service, 1950) is now in effect state-wide in 13 states, as well as in 360 counties and 1,464 municipalities located in 39 states. It has been adopted as a regulation by 34 states and territories.

## IN-PLANT CHLORINATION

In-plant chlorination of processing water is employed in many food industries including cannery, freezing, and poultry dressing plants. In many of these plants it is the common practice to employ break-point chlorination. In this method of chlorination, the typical flavors and odors associated with chlorinated water are first produced and then as chlorination increases the chlorine residual decreases as the result of a chemical reaction between the added chlorine and the chloramines produced in the initial step of chlorination. When this reaction is complete, the addition of more chlorine increases the residual in proportion to the applied chlorine. This is known as the break-point in the residual chlorine curve. At this point the objectionable tastes and odors have been greatly reduced or entirely disappeared and as chlorination continues, a chlorinous taste develops.

In the cannery and freezing plants the in-plant chlorination of processing water aids in reducing bacterial contamination throughout the plant, and makes it easier to remove such slime that may have developed on plant equipment. This is due to the fact that such slime as may have developed does not adhere so tenaciously and enables the clean-up crew to do more thorough work in a shorter time period. Because of the difference in chlorine demand of waters, it becomes necessary to determine the break-point on the untreated water in the laboratory before application to the plant's entire water supply.

In poultry dressing plants, Goresline, *et al.*, (1951) reported that the use of break-point chlorination in processing water by maintaining 10 to 20 ppm free residual chlorine markedly improves the sanitary quality of the dressed poultry, reduces the bacterial population at every point of contact and prevents the development of slime bacteria on equipment and in pipe lines, reduces odors in the plant and reduces about one-third of the time necessary for plant clean-up.

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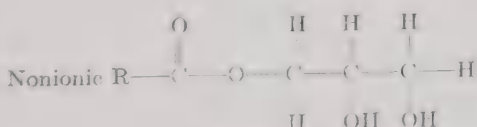
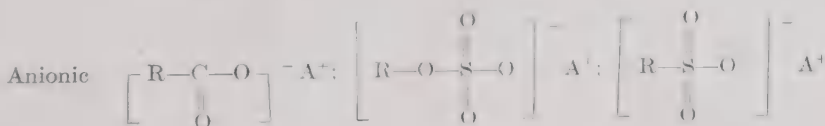
## 23

# QUATERNARY AMMONIUM COMPOUNDS

## GENERAL INTRODUCTION AND CHEMISTRY

THIS chapter could more appropriately be titled "Quaternary Ammonium Surface-Active Disinfectants" inasmuch as only those compounds will be discussed here which have long chain alkyl, or heterocyclic, groups (usually derived from corresponding long chain alkyl groups obtained from vegetable or animal fatty acids) in the cation portion of the molecule. These compounds may, therefore, be considered "surface-active" in the sense that they have the property of lowering the surface tension of solutions.

The relationship of the "cationic" surface-active quaternary ammonium germicides (Quats) to the other two groups of synthetic surface-active agents, anionic and nonionic, may be illustrated as follows:

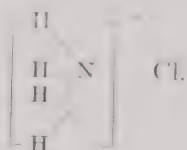


R represents a lipophilic group such as long chain alkyl or polycyclic, *i.e.*, cholesteryl, aryl-alkyl radical, etc., and including



derivatives of these groups. A represents a positive ion such as Na, K,  $\text{NH}_4$ , etc. The groups to which R is attached are the "hydrophile" portions of the molecule.  $\text{X}^-$  represents a negative ion and  $\text{R}_1, \text{R}_2, \text{R}_3$  represent H, alkyl, aryl or heterocyclic groups or residues. Young and Coons (1945) have prepared a comprehensive account on the theoretical aspects and application of the various classes of synthetic surface-active agents or "detergents."

The surface-active quaternary ammonium germicides (Quats) belong to a special class of chemical disinfectants characterized as a group of amines which can be synthesized to give nitrogen compounds with the latter radical having a covalence of 5. An example of a simple salt of this nature is ammonium chloride. With the

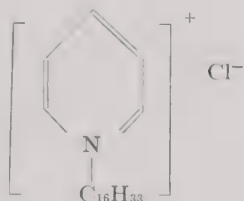
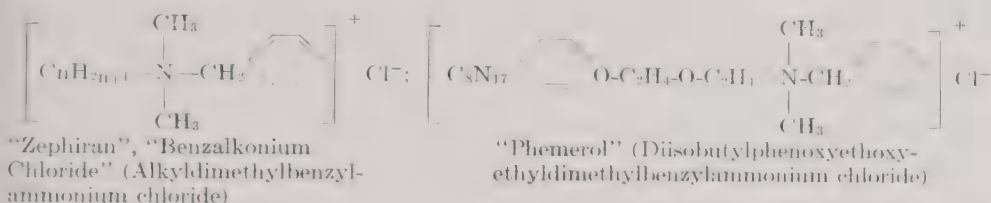


Quats, however, the hydrogen atoms in the latter compound are replaced with one or more alkyl groups ( $\text{CH}_3$ ,  $\text{C}_2\text{H}_5$ , etc.) or a phenyl radical, and one of the hydrogens substituted by an alkyl group containing  $\text{C}_8$  to  $\text{C}_{18}$  carbon chain lengths. The surface activity of these germicides is due to the latter groups which are derived from natural vegetable or animal oils and resins.

Since the introduction of the Quats as general and medicinal disinfectants by Domagk (1935), these chemical agents have received wide and popular recognition as effective germicides for the destruction of many forms of germ life. The inherent features of the compounds which make them useful as disinfectants are that they contain no phenol, iodine, active chlorine, mercury or other heavy metals. They have no odor, color, are highly stable and are nontoxic when used in recommended concentrations. They appear to be equally effective against many of the gram-positive and gram-negative bacteria that are encountered in general and medical disinfectant practices.

Evidence of the interest shown in the Quats as disinfectants may be had from the 800 or more publications that have appeared in various journals during the past two decades in which mention is made of the use of this class of chemical disinfectants. Three of the Quats are listed in the 1949 edition of New and Nonofficial Remedies (Zephiran, Phemerol, Ceepryn) and one appears in the 13th edition of the United States Pharmacopeia (Benzalkonium

Chloride). The chemical structures of these compounds are as follows:



"Ceepryn" cetylpyridinium chloride

Insofar as the antibacterial activities of the three groups of surface-active detergents are concerned (cationic "Quats," anionic, nonionic), the cationic type of compounds are almost equally effective against both the gram-positive and the gram-negative bacteria. The anionics, on the other hand, are mainly active against the gram-positive organisms and the nonionics are considered to have little, if any effect on both groups of bacteria. Furthermore, there exists a definite "antagonistic" or "interfering" action between the cationic and anionic types of surface-active agents; one "inactivating" the germicidal activity of the other (Lawrence, 1948). The nonionic group of compounds do not ionize in aqueous solutions and probably for this reason they do not interfere with the antibacterial activities of either of the other two classes of surface-active agents. Since some of the nonionic compounds are excellent solubilizing and "foaming" agents, they are often added in formulations with the cationics and other chemical compounds where "true" solutions and "germicidal-detergent" combinations are desired.

Although the early literature (Einhorn and Göttler, 1908; Mannich and Hahn, 1911; Reychler, 1913) described the synthesis and certain limited references of the antimicrobial activity of the Quats (Einhorn, *et al.*, 1905), their use as antiseptics for general and surgical disinfection was not widely recognized up to the time of their introduction by Domagk (1935). Within the same year approximately 25 articles appeared in foreign journals on the successful use of the Quats in surgical disinfection. One of the early publications on the use of one of the compounds in the United

States was the result of the studies of Dunn (1936). The historical development of these compounds with regard to their chemistry, physical properties, biological activities, and application as antiseptics has been reviewed by Lawrence (1950) and to some extent by Glassman (1948).

While the three classes of surface-active agents (cationics, anionics, nonionics) are very extensively used in the cosmetic, tanning, ore-flotation, dyeing, oil, adhesive, food and related industries (Young and Coons, 1945), reference in this chapter to the cationic (Quat) class of compounds will be made mainly on their application as antimicrobial agents.

### MODE OF ANTIMICROBIAL ACTION

The higher forms of organisms (protozoa, invertebrates, etc.) have been found to be markedly altered in their cellular morphology by Quat treatment and accordingly may explain one of the modes of action of this class of germicides as being due to surface activity on living cells (Taft and Strandtmann, 1945; Taft, 1945, 1946). However, the normal structure of bacteria and fungi appears to be unaffected by Quats and other surface-active agents as evidenced by the lack of change in cell morphology noted under the ordinary microscope. Thus, much of the work on determining the mode of action of Quats on bacteria, fungi and viruses involves consideration of failure of the treated organisms to grow in certain media, or failure of the pathogenic varieties to produce disease, and/or death in a susceptible laboratory animal.

A number of investigators have used bacterial respiration studies for determining the effects of Quats and other germicides upon the metabolism of microorganisms and used this criterion for the evaluation of the destructive action of the compounds upon living cells (Miller, Baker, and Harrison, 1939; Jerchel, 1943; Ordal and Borg, 1942; Sevag and Ross, 1944). In general the Quats have been found to have an effect upon certain organisms by interfering with respiration and glycolysis of the bacteria with particular effects of inhibiting the oxidation of certain carbohydrates.

Hotchkiss (1946), on the other hand, found that upon treating certain bacteria with Quat solutions and other surface-active germicides, a release of nitrogen and phosphorous from the cells into the surrounding medium would occur. Many of the other germicides, lacking surface-activity (mercuric chloride, acridine dyes, active chlorine, etc.), failed to cause a similar leakage of the cellular element from the same organisms. Gayle and Taylor (1946) identified



some of the substances released by tyrocidin\*-treated cells as being free lysine and glutamic acid. The several other concepts relative to the mode of action of Quats upon microorganisms (*viz.* enzymes, proteins, nucleic acid, toxin production, etc.) have been reviewed to some extent by Albert (1942) and Lawrence (1950a).

## ANALYSES FOR QUATERNARY AMMONIUM CONCENTRATIONS

The chemical constitution of Quats is such that they lend themselves readily to combination with several dye indicators, formation of a precipitate with other surface-active agents (anionics), or precipitation in the presence of certain organic substances. Some of these reactions are quantitative and have been used to determine small amounts of the germicides in solutions under laboratory and in conditions of actual use.

Hartley and Runnicles (1938) were probably the first to make use of a chemical test for determining Quat concentrations by use of a colorimetric procedure. The use of methods in which chemical reagents will quantitatively precipitate the germicides have been described by Auerbach (1947), Bartlett (1937) and Flanagan, Drennen and Goetchius (1948). A few tests have been described in which both a color and a precipitate are formed by combining certain chemical reagents with Quats (DuBois, 1945; Harper, Elliker and Moseley, 1948). Probably the most popular methods for determining Quat concentration under conditions of use are the "test-papers" consisting of strips of filter paper which are impregnated with certain sulfonothalein dyes (Stone, 1947; Portley, 1949).

Gain and Lawrence (1947) described a test method for determining "use concentrations" of Quats in which normal horse serum was used as the test reagent. Mention may also be made of a unique procedure for determining bactericidal concentrations of Quats, as well as chlorine disinfectants, in which an actively motile culture of *Escherichia coli* is used as the test medium (Buchbinder and Zaretsky, 1951). Cessation of motility of the microorganisms is taken as an indication of the lethal concentration of the disinfectant solution under examination.

While many of the chemical methods will give accurate determinations of Quat solutions under controlled conditions in the

Tyrocidin is apparently a surface-active antibiotic and part of its antimicrobial activity may be attributed to this property. Two other antibiotics, subtilin (Anderson, 1947) and penicillin (Hauser, Phillips and Vavrukh, 1948), have also been shown to possess surface tension reducing action in solutions.

laboratory, there is still need of a procedure that can be relied upon to give some degree of accuracy when the tests are conducted in the field, when Quats are diluted with certain natural waters known to have an adverse effect on the germicidal activity of the disinfectants (U. S. Public Health Service, 1947; Ridenour and Armbruster, 1948; Lawrence, 1950; Johns, 1947, 1948; Mueller and Seeley, 1948). Thus, the motility test of Buchbinder and Zaretsky appears to give a more exact method for determining the effective killing concentrations of Quat solutions under actual use conditions.

## COMPATIBILITIES AND INCOMPATIBILITIES

Several publications have listed a variety of compatibilities and incompatibilities of various chemical compounds with Quats (Huyck, 1944; Lawrence, 1950). Of particular importance are the incompatibilities that will occur under practical conditions of the use of this class of disinfectants. Domagk (1935) was first to call attention to the interfering action of ordinary soap (an anionic surface-active agent) upon the germicidal activity of a Quat solution. It is generally recognized, therefore, that when surfaces have been washed with ordinary soap and water, the objects must be thoroughly rinsed with water to remove any residual soap before applying a Quat for sterilizing purposes. This applies also to the use of the disinfectant following a cleansing procedure in which the synthetic surface-active anionic detergents are used.

An incompatibility of present and important concern exists between Quats and some substance or substances present in certain natural waters, to which reference was made in the previous section of this chapter. Probably the the first suggestion of the actual substances present in some well waters that interfered with the germicidal activity of Quats may be found in the publication of Ridenour and Armbruster (1948). These investigators compared the efficiencies of several Quats that were currently on the market; the effects of temperature, organic matter, water mineral substances and pH on the germicidal activity of the compounds; relative resistance of various types of organisms to the bactericidal agents; and chemical testing for activity of "residual" quaternary ammonium concentration under various conditions of use. Suffice it to say that these studies revealed that cations such as calcium and magnesium, normally considered responsible for the "hardness" of waters, will materially reduce the germicidal activity of Quats. A decrease in bacterial count from normal of 99.9 to 50 per cent may occur with an increase of calcium or magnesium cations from 0 to 40 parts per million of water. On the other hand, sodium and potas-

sium cations, as well as the several anions they tested, failed to have any adverse effect on the Quats they studied.

While the U. S. Public Health Service (1947) had presented data early on the adverse effects of some municipal water supplies on Quats, they failed to indicate the nature of the substances that were responsible for this activity. Nevertheless, on the basis of their studies they suggested that health officers could provisionally permit the use of Quats in certain sanitary procedures but only on a limited scale and here under controlled conditions where frequent bacteriological sampling could be carried out. As a result of this regulation the Weber and Black (1948) laboratory testing procedure has been advocated for evaluating the practical activity of Quats and other disinfectants that are used for the treatment of food utensils.

Although Ridenour and Armbruster indicated that calcium and magnesium cations in hard waters were responsible for the interfering action on Quats, Mueller and Seeley (1951) noted that waters with relatively small amounts of ferrous and ferric ions were much more detrimental to the activity of this class of compounds. Furthermore, there is some evidence to indicate that some form of aluminum may be the substance present in certain waters which causes the adverse action on most Quats. An interesting aspect on this subject is the observation of Dennis (1951), who found that the germicidal properties of a Quat were not reduced below a required concentration of 200 parts per million even in the presence of a natural water with a high degree of hardness.

Studies by Johns (1947, 1948) and Lawrence (1950) revealed that while many of the Quats were reduced in activity when a gram-negative bacterium (*E. coli*) was used as the test organism in the presence of "hard" water, the same germicides retained their activity when a gram-positive organism (*Micrococcus pyogenes* var. *aureus*) was tested in the same water. It appears doubtful whether a Quat can be synthesized which will be entirely unaffected by various natural waters. A possible approach to this problem appears to be in the use of certain chelating agents to counteract the interfering agents in waters (Peabody and Mallmann, 1952) and the use of Quat-detergent (polyphosphates, etc.) mixtures (Hucker, 1947).

## PHENOL COEFFICIENT AND USE DILUTION TESTS

It is doubtful whether the pages of this text could accommodate the details of the many test procedures that have been described and advocated for testing the antimicrobial activity of Quats. The



several anomalies that appear in the "Phenol Coefficient" (U. S. Dept. Agriculture, 1931) testing of this class of disinfectants are apparent to investigators evaluating these compounds. Some of the variations appear due to the inconsistency in the composition of the culture media (Brewer, 1943; Wolf, 1945; Klarmann and Wright, 1945); unreliability in the use of the platinum loop for transferring uniform amounts of the treated organism suspension from the Quat test solution (Klarmann and Wright, 1946; Tobie and Ayres, 1944); the adherence of the Quat to the walls of test tubes along with bacteria due to the electrical charge imparted by the germicide, thus giving fictitious results in the test (Klarmann and Wright, 1946); an "agglutinating" action of the Quats upon microorganisms which results in an "apparent" reduced bacterial count in agar medium (Klarmann and Wright, 1946a); and a "coating" effect of the Quats upon microorganisms which prevents them from multiplying in the subculture media (McCulloch, Hauge, and Migaki, 1948; McCulloch, 1947, 1947a). For the reasons given, a wide variety of modified Phenol Coefficient and other test procedures have been described for evaluating the germicidal activity of Quats.

This presentation of the various factors that may be responsible for variations in the phenol coefficient testing of a specific class of germicides will indicate the sources of error that can be expected in a single testing program. At most the laboratory "test tube" methods give no more than a suggestion as to the germicidal activity of the same compound which will be actually used in general and medicinal disinfectant practices. Nevertheless, having once established the relative merits of a disinfectant under use conditions, one could logically use a simple phenol coefficient or "Cationic Coefficient" test to check the same compound in the laboratory under carefully controlled conditions.

In Table 91 are tabulated a number of phenol coefficient values that have been reported by several investigators on Quat solutions. From the data given in the table it is evident that the Quats can be expected to give high phenol coefficient values against the two organisms listed, *M. pyogenes* var. *aureus* and *Salmonella typhosa*. With but few exceptions, the phenolics (Coulthard, Marshall, and Pyman, 1930; Suter, 1941) and mercurial compounds (Deskowitz, 1937) give much lower values in the same test procedures.

"Use Dilution" techniques have been employed by many investigators for evaluating the Quats and other classes of disinfectants in which it is felt that the conditions of testing simulates more closely the use for which the germicides are recommended. In general the methods make use of various "carriers" in the form of

glass rods (Mallmann and Hanes, 1945), glass rings (Stuart, 1947), glass strips or slides (Klarmann and Wright, 1946; Johns, 1947a), linoleum, asphalt, rubber, plastic or porcelain floor tiles (Klarmann, Wright and Shternov, 1953) and other materials. In comparing the persistence of the antibacterial properties on a variety of floor materials, Klarmann and his coworkers found that the

TABLE 91. — PHENOL COEFFICIENTS ON QUATERNARY AMMONIUM GERMICIDES  
(LAWRENCE, 1950)

Quaternary	Author	<i>S. aureus</i>		<i>E. typhosa</i>	
		20°	37°	20°	37°
Alkyldimethylbenzylammonium Cl	Kliewe, 1936	150	—	200	—
Alkyldimethylbenzylammonium Cl	Dunn, 1936	279	407	250	429
Alkyldimethylbenzylammonium Cl	Heineman, 1937	275	293	176	271
Alkyldimethylbenzylammonium Cl	Deskowitz, 1937	362	324	317	310
Alkyldimethylbenzylammonium Cl	Maier, 1939	150	—	200	—
Alkyldimethylbenzylammonium Cl	Lawrence, 1946	250	325	275	314
p-tertiaryocetylphenoxyethoxyethyl- dimethylbenzylammonium Cl	Lawrence, 1946b	323	300	200	257
9-octadecenyl dimethylethylammonium Br	Lawrence, 1946b	250	262	133	228
Cetylpyridinium Cl	Lawrence, 1946b	350	337	133	228
N-(acylcolaminoformylmethyl) pyridinium Cl	Lawrence, 1946b	150	200	111	200
N-(acylcolaminoformylmethyl) pyridinium Cl	Epstein, 1943	500	571	222	343
Cetyltrimethylammonium Br pH 5.0	Hoogerheide, 1945	300	650	—	—
Cetyltrimethylammonium Br pH 6.7	Hoogerheide, 1945	500	1,000	—	—

Quats and several other types of disinfectants would maintain a definite germicidal activity against a number of pathogenic bacteria when the germicides were tested on porcelain tiles. However, many of the compounds tested rapidly lost their ability to destroy micro-organisms when they were applied to linoleum, rubber, plastic, etc., surfaces.

In the "Use Dilution" tests that have been described by various workers the general procedure consists of applying the test organisms to the surface of materials in the form of broth suspensions of the bacteria, or the latter were first suspended in some organic matter and then applied to the objects. The culture is permitted to dry on the surface of the latter for varying periods of time and then dipped into solutions of the disinfectant under test. The excess of disinfectant is drained from the treated materials and the presence or absence of viable organisms remaining on the material determined by adding the latter to sterile broth, or the surface is swabbed with a cotton applicator and inoculated into nutrient media. The appearance or lack of appearance of turbidity in broth and presence or absence of colonies to develop in agar are taken as indications of the relative efficiency of the disinfectant

upon the contaminated object under test. Table 92 presents the results of a comparison of the phenol coefficient test and a "Glass Ring Carrier" procedure which has been described by Stuart (1947).

TABLE 92.—COMPARISON OF "USE DILUTION" AND PHENOL COEFFICIENT (F.D.A.) METHODS FOR TESTING QUATERNARY AMMONIUM GERMICIDES (LAWRENCE, 1950)

Quaternary	Dilution	<i>S. aureus</i> Transfer Min.			Phenol Coeff.	<i>E. typhosa</i> Transfer Min.			Phenol. Coeff.
		5	10	15		5	10	15	
I. Glass rings used as "carriers" for subculture to a quaternary inactivating medium.*									
"B"†	1:9,000	—	—	—	14,000 = 187	—	—	—	15,000 = 95
	1:12,000	—	—	—		—	—	—	
	1:15,000	+	+	—		+	—	—	
	1:18,000	+	+	—		+	+	—	
	1:21,000	+	+	+		+	+	—	
	1:24,000	+	+	+		+	+	—	
"C"†	1:27,000	+	+	+	9,000 = 128	+	+	+	9,000 = 95
	1:6,000	+	—	—		—	—	—	
	1:9,000	—	—	—		+	—	—	
	1:12,000	+	+	—		+	+	+	
	1:15,000	+	+	—		+	+	+	
	1:18,000	+	+	—		+	+	+	
"E"†	1:21,000	+	+	+	15,000 = 214	+	+	—	11,000 = 95
	1:6,000	—	—	—		—	—	—	
	1:9,000	—	—	—		—	—	—	
	1:12,000	+	—	—		+	+	—	
	1:15,000	+	—	—		+	+	—	
	1:18,000	+	+	+		+	+	—	
II. Standard 4-mm loop used for subculture to a quaternary inactivating medium.*									
"B"	1:12,000	—	—	—	21,000 = 323	—	—	—	21,000 = 90
	1:15,000	+	—	—		—	—	—	
	1:18,000	—	—	—		+	—	—	
	1:21,000	+	—	—		+	—	—	
	1:24,000	+	+	—		+	+	—	
	1:27,000	+	+	+		+	+	+	
"C"	1:9,000	+	—	—	15,000 = 230	—	—	—	12,000 = 90
	1:12,000	+	—	—		+	—	—	
	1:15,000	+	—	—		+	+	—	
	1:18,000	+	+	—		+	—	—	
	1:21,000	+	+	—		+	+	—	
	1:24,000	+	+	+		+	+	—	
"E"	1:9,000	—	—	—	15,000 = 230	—	—	—	15,000 = 90
	1:12,000	+	—	—		—	—	—	
	1:15,000	+	—	—		+	—	—	
	1:18,000	+	+	—		+	+	+	
	1:21,000	+	+	—		+	+	+	
	1:24,000	+	+	—		+	+	+	

\* Modified Lethcen Broth.

† "B" = Alkyldimethylbenzylammonium chloride. "C" = Cetylpyridinium chloride.  
"E" = p-tertiaryoctylphenoxyethoxyethylmethylbenzylammonium chloride.

## GERMICIDAL ACTIVITY OF QUATS IN PRESENCE OF ORGANIC MATTER

A number of workers have noted that some of the Quats were more active than many other germicides when tested in the presence



f serum proteins (Domagk, 1935; Deskowitz, 1937; Kliewe and Maier, 1936). Hornung (1935) reported that a 0.01 per cent solution of a Quat, in the absence of serum would destroy *M. pyogenes* var. *aureus* in 15 minutes and a 0.03 per cent of the germicide would do the same in the presence of 16 per cent serum. In a similar test Schneider (1935) found that 10 per cent blood serum would reduce the germicidal activity of the compound by only 10 per cent.

TABLE 93.—LIMITING DILUTIONS OF QUATERNARY AMMONIUM COMPOUNDS EFFECTIVELY KILLING OR INHIBITING GROWTH OF *S. aureus* IN BROTH MEDIUM (LAWRENCE, 1950)

Quaternary	Author	<i>S. aureus</i>	
		Bc*	Ps*
Trimethylammoniumphenol-n-dodecylether methosulfate	Kuhn, 1940	1:4,800	1:19,200
Trimethylammoniumphenol-n-dodecylether methosulfate	Kuhn, 1940	1:4,800	1:19,200
Trimethylammoniumphenol-n-dodecylether methosulfate	Kuhn, 1940	1:1,600	1:25,600
Dodecyltrimethylbenzylammonium Br	Kuhn, 1940	1:1,200	1:12,800
Alkyldimethylbenzylammonium Cl	Jerchel, 1942	1:38,400	1:200,000
Diphenylundecyltetrazolium Cl	Schneider, 1935	—	1:409,600
4-phenyl-3-a-naphthyl-5-n-undecyltetrazolium Br	Kuhn, 1941	1:38,400	1:76,800
4-phenyl-3-n-bromophenyl-5-n-undecyltetrazolium Br	Kuhn, 1941	1:2,400	1:4,800
Trimethylammonium Br	Williams, 1943	1:218,700	—
Trimethylpyridinium Br	Williams, 1943	1:218,700	—
Alkyldimethylbenzylammonium Cl	Deskowitz, 1937	—	1:320,000
Alkyldimethylbenzylammonium Cl	Lawrence, 1946	1:200,000	1:800,000
Decyltrimethylammonium Br	Hoogerheide, 1945	—	1:7,000
Dodecyltrimethylammonium Br	Hoogerheide, 1945	—	1:70,000
Tetradecyltrimethylammonium Br	Hoogerheide, 1945	—	1:340,000
Trimethylammonium Br	Hoogerheide, 1945	—	1:400,000

\* Bc = Bactericidal.

Bs = Bacteriostatic.

Since the Quats are used extensively for the sterilization of milk processing equipment several investigators have studied the effects of milk upon the germicidal activity of Quats. While McCulloch, Hauge and Magaki (1948) noted that 50 per cent of milk will markedly reduce the germicidal properties of a Quat, Johns (1948) was able to show that a 99 per cent kill of *M. pyogenes* var. *aureus* can be expected of a Quat within 15 seconds in the presence of 10 per cent skim milk. A consideration of the detection of and effects of Quats on microorganisms has been undertaken by Dubois and Dibblee (1946), Johns and Pritchard (1946), and Mull and Gouts (1947).

The use of the conventional (U. S. Dept. Agriculture, 1931) Agar Cup-Plate method for determining the activity of Quat-containing solutions, ointments, etc., is contraindicated. This is based on the fact that several investigators have found that the agar in

the test media will quantitatively adsorb this class of chemical disinfectants and thus lead to erroneous results. Tobie and Ayers (1944) attributed the discrepancies in the agar plate tests to dif-

TABLE 94.—LIMITING DILUTIONS OF SEVERAL ALKYLDIMETHYLBENZYLAMMONIUM CHLORIDES SHOWING ANTIBACTERIAL EFFECTS IN NUTRIENT MEDIA (LAWRENCE, 1950)

Organism—Strain	Compound—Dilution*											
	I†		II		III		IV		V		VI	
	Bs‡	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
<i>E. typhosa</i> "H"	256	128	256	128	16	8	128	128	128	128	16	16
<i>S. dysenteriae</i> "F"	512	256	128	32	32	8	256	128	256	128	16	8
<i>E. coli</i>	64	32	256	128	4	4	128	32	64	16	4	2
<i>A. aerogenes</i>	16	16	32	16	1	<1§	32	32	32	16	<1	<1
<i>S. paratyphi</i>	64	32	32	32	4	2	64	32	32	32	1	1
<i>S. enteritidis</i>	32	32	16	16	4	2	32	32	32	32	<1	<1
<i>Proteus vulgaris</i>	16	8	16	16	8	4	16	16	32	16	128	4
<i>Ps. aeruginosa</i> "I"	32	32	64	16	1	1	64	64	64	32	<1	<1
<i>V. cholera</i> "As"	512	256	256	128	64	16	256	64	256	128	4	4
<i>S. aureus</i>	800	200	400	200	50	50	200	100	800	100	50	25
<i>Pneumo. II</i>	200	200	800	400	800	400	800	400	800	200	400	400
<i>S. pyogenes</i>	800	200	1,600	400	800	400	800	400	1,600	400	800	400
<i>C. l. welchii</i>	200	200	400	400	25	25	800	800	200	200	50	50
<i>C. l. tetani</i>	200	200	400	200	100	25	800	800	400	400	100	50
<i>C. l. histolyticum</i>	200	50	400	400	100	25	800	800	800	200	50	25
<i>C. l. oedematiens</i>	200	200	200	200	25	25	800	800	50	50	50	50

\* All values given represent dilutions in thousands, i.e., 256 = 1:256,000, etc.

† I lorolbenzyl, II cetylbenzyl, III octadecylbenzyl, IV dodecylbenzyl, V myristyl-3,4-dichlorbenzyl, VI cetyl-1,3,4-dichlorbenzyl.

‡ Bs = compound-dilution bacteriostatic; Bc = compound-dilution bactericidal.

§ Figures preceded by < indicates the highest concentration of cationic compound tested and failed either to kill or inhibit the growth of the test organism.

ferences in the rate of diffusion of the Quats in the solid medium. Quisno, Gibby and Foter (1946) on the other hand, found that the variations were the result of a quantitative physical absorption of the Quats on the agar itself. A similar lack of agreement between

broth dilution and agar plate tests has been noted with chlorine disinfectants (Mudge and Smith, 1935).

An indication of the activity of Quats upon microorganisms in the presence of small amounts of organic matter may be had from the various reports in which the germicides were tested against bacteria in broth media. In carrying out these tests the procedure usually involves the addition of Quat dilutions to nutrient broth to which are then added the test organisms. The tubes are incubated for several days and the presence or absence of growth noted. To distinguish between bacteriostasis and bactericidal activities, subcultures are made from the tubes to additional tubes containing plain nutrient media or to agar plates. The latter are incubated further in order to detect viable organisms. Tables 93 and 94 present a series of results obtained in the manner indicated.

### INFECTION-PREVENTION TESTS

Test procedures have been described for the evaluation of disinfectants in which the treated, pathogenic organisms are injected into susceptible animals rather than inoculating the cultures in nutrient laboratory media. Multiplication of the organisms in the animals, with an accompanying appearance of the former in the blood or tissues and even death of the animals is taken as an indication that the culture was not destroyed by the germicide. On the other hand, if the animals survived and no organisms were found in the tissues after the former were sacrificed at the termination of the experiment, the disinfectant under test was considered lethal for the original organism suspension.

Neufeld and Schutz (1943) and later Dennis (1951) exposed cultures of *Salmonella* and *Pasteurella* to Quat solutions and then injected the organisms into mice. In both instances relatively dilute solutions of a Quat were found to kill or nullify the infectivity of the organisms following short periods of exposure to the germicide. The high antibacterial activity of a Quat against *Salmonella* bacteria in mouse test procedures was also reported by Kenner, Quisno, Gibby and Foter (1946).

Nungester and Kempf (1942), Pierce and Tilden (1946), and Spaulding and Bondi (1947) found that the concentrations of aqueous solutions of Quats ordinarily recommended for skin disinfection were relatively ineffective in destroying mouse-virulent strains of pneumococci as evidenced by the high incidence of mortality of the animals injected with the treated organisms.

Several investigators have used the developing chick embryo for determining the infectivity or survival of bacteria after the



latter had been exposed to disinfectants. Green (1944), Green and Birkeland (1942), and Gershenfeld and Witlin (1947) used *M. pyogenes* var. *aureus* in their studies and found that the Quats were effective in destroying the latter organisms as evidenced by the survival of the chick embryo from infection with the bacteria. A comparison of the relative toxicities of the disinfectants under examination was also made in the chick embryo studies indicated.

### SANITIZING TESTS

The term "sanitize" denotes a process or condition in the treatment of objects to minimize the possible transmission of disease with particular reference in public health in controlling sanitary conditions in eating and drinking establishments, in the dairy industry and other food processing plants. Thus, the term "sanitize" implies a process of disinfection "without any residue harmful to subsequent users of the article or product, as well as the elimination of the contamination which might be esthetically objectionable (McCulloch, 1945).

Evidence to the effectiveness of Quats as "sanitizing" agents in the treatment of utensils may be had from various publications in which the compounds were compared with chlorine disinfectants under actual working conditions (Krog and Marshall, 1940, 1942; MacPherson, 1944; Mallmann, Kivella and Turner, 1946, etc.).

### GENERAL ANTIMICROBIAL ACTIVITY OF QUATS

Much of the information on the antibacterial activity of Quats is concerned with tests on the compounds in which gram-negative bacteria and gram-positive, nonsporeforming organisms have been used. Yet there are a number of important articles describing the activity on other specific groups of microorganisms of which brief mention will be made at this point.

In those instances where a sporicidal activity of Quats has been noted one might expect that aqueous concentrations greater than that found to be effective against nonsporeformers are required for the former bacteria (Domagk, 1935; Hornung, 1935; Nagel, 1940, etc.) By varying the pH or temperature of the test suspensions the Quats could be demonstrated to have an enhanced effect upon spores of microorganisms (Sabalitschka and Maas, 1939; Dubois and Dibblee, 1946a, etc.).

The concentrations of aqueous Quats ordinarily recommended for the sterilization of surgical instruments (1:1000) will not destroy human strains of the tubercle bacilli in short periods of

exposure (Domagk, 1935; Smith, *et al.*, 1950; Lawrence and Grover, 1951). On the other hand, several of the Quats have been found to exhibit a definite degree of inhibition of growth of the same organisms in dilutions ranging from 1:80,000 to 1:1,000,000 (Freedlander, 1940; Lawrence, Wilson and Goetchius, 1945). The saprophytic acid-fast bacteria are also sensitive to the inhibitory action of Quats since concentrations of 1:2,000 to 1:4,000,000 in broth have been found to completely suppress the growth of these organisms (Katz and Lipsitz, 1937; Quisno and Foter, 1946; Lawrence *et al.*, 1945).

TABLE 95.—ACTION OF A 1/1000 TINCTURE\* OF ALKYLDIMETHYLBENZYLAMMONIUM CHLORIDE AGAINST SOME FUNGI (DUNN, 1938)

	Average—Highest dil. destroying organism in 10 but not 5 minutes§					
	Phenol	Coeff.		Ammonium compound	Phenol	Ammonium compound
Test organisms	20° C	37° C	Phenol 20° C		37° C	
Trichosporon schoenleinii	0.2	0.36†	1/87	1/17	1/140	1/50
Trichomyces gelanesis	1.0	1.3	1/130	1/130	1/205	1/275
Coccocus hominis‡	0.21	0.4	—	1/24	—	1/70
Trichophyton inguinale	0.36	0.44	1/123	1/45	1/167	1/73
Sporon audouini	0.074	0.12†	1/175	1/13	1/320	1/35
Sporon lanosum	0.089†	—	1/90	1/8	—	—
Candida albicans	0.17	0.60	1/77	1/13	1/93	1/55
Trichomyces cerevisiae	0.09	0.15	1/100	1/9	1/135	1/21
Trichophyton interdigitale 1	0.1	0.28†	1/60	1/6	1/90	1/25
Trichophyton interdigitale 2	0.12	0.28†	1/63	1/7	1/90	1/25

\*Alcohol-acetone-aqueous solution

†One test only

‡Figures based on 1/1000 aqueous solution of the ammonium compound

§Dilutions of the 1/1000 solution

Dunn (1936) described the antifungal activity of a Quat against a variety of fungi in which phenol coefficient values were obtained from 11 through 400 against several of the pathogenic varieties. A subsequent report by the same investigator indicated the effects of a tincture of a Quat on a number of fungi, the data of which are reproduced in Table 95 (1938). The fungicidal activity of Quats on a miscellaneous group of fungi has also been described by Heineman (1937), Joslyn, Yaw and Rawlins (1943), Quisno and Foter (1946), Lawrence (1946), Knight and Frazier (1945) and others.

Quats have also been found to be effective against a number of pathogenic protozoa. Fair and his coworkers (1945), and Kessel and Moore (1946) noted that concentrations ranging from 1:5,000 to 1:80,000 of some Quats would destroy the cysts of *Endamoeba*

*histolyticum*. The latter investigators suggested the possible use of a Quat in the emergency sterilization of drinking water supplies. Moore and Marmorston (1946) later described methods for the removal of the objectionable, bitter taste of Quat-treated waters with particular reference to the use of colloidal clays.

An indication of the sensitivity of other protozoa to Quats may be had from the work on *Trichomonas vaginalis* (Johnson and Trussell, 1943; Quisno and Foter, 1946) and *Tr. foetus* (Morgan and Campbell, 1946; Lawrence, 1946a). Concentrations of a Quat as low as 1:30,000 have been found to destroy the latter organism. Reference has already been made in an early section of this chapter to the studies of Taft and Strandtmann in which Quats were found to be highly effective in destroying a wide variety of invertebrates.

Viruses in general appear to be somewhat more resistant than bacteria and fungi to the lethal effects of Quats. This fact has served as a useful means of preparing bacterial-free vaccines of virus suspensions (Kaiser, 1937; Ducor, 1947; U. S. Navy, 1942, 1946). The relative ineffectiveness of Quats upon purified strains of viruses, as compared to the high germicidal activity of the compounds upon many bacteria, probably contraindicates the use of this class of disinfectants on suspected virus-contaminated materials.

### TOXICITY OF QUAT DISINFECTANTS

Chronic and acute toxicity studies in animals, and an occasional report on the irritating effects on human skin, indicate that the various Quats exhibit almost identical toxicological and pharmacological properties (Becker *et al*, 1940; Nelson and Lyster, 1946; Woodward and Calvery, 1945; Alfredson *et al*, 1951; Kylin, 1949, 1949a; etc.). In general the toxic reactions noted by giving a Quat solution by the intravenous route and in lethal doses, results in convulsion or depression prior to death. The latter is due to asphyxia from paralysis of the respiratory muscles very similar to poisoning from curare. High concentrations (3 per cent) of a Quat applied to the freshly shaved skin of rabbits have been found to cause inflammation, edema and scar formation. Yet the concentration usually recommended for use on the normal skin (1:1,000 to 1:5,000) can be expected to cause little or no irritation or discomfort to the animal body. The inclusion of as much as 0.3 per cent of a Quat in the diet of animals has been found to be the maximal tolerated dose for animals in which no toxic effects can be noted. Animals given a 1:1,000 solution of a Quat as their only source of drinking water appeared to tolerate the disinfectant for several months without any impairment in weight gain or other untoward



reactions. On the basis of these observations and the wide use of Quats in eating and drinking establishments, it is doubtful that any trace amounts of the disinfectants left on the utensils would result in any accumulative effect upon the users of the objects. Yet, for precautionary measures instructions are usually given for a final rinse of utensils in running tap water following treatment with a Quat disinfectant. There has been one death of a human being who accidentally swallowed a relatively large amount (1 oz) of a 10 per cent solution of a Quat (Adelson and Sunshine, 1952).

## PRACTICAL APPLICATION OF QUATS

*Eating and Drinking Establishments, Dairies and Food Plants, Etc.*—In the original publication of Domagk (1935), in which a Quat was recommended for general use as a disinfectant in hospital practices, conspicuous by its absence was the presentation of any data on bacterial counts of areas or surfaces that had been treated with the disinfectant. Probably one of the first reports presenting the germicidal efficiency of a Quat as a sanitizing agent in eating and drinking establishments appeared in an article by Krog and Marshall (1940). Subsequent data of other workers substantiated the findings of the latter investigators and also supplemented the advantages on the use of Quats in restaurant sanitation (Walter and Hucker, 1942; MacPherson, 1944; Mallmann *et al*, 1946; Botwright, 1946; etc.).

Krog and Marshall (1942) also advocated the use of Quats in the disinfection of equipment used in the dairy industry. The compounds have been found effective in sterilizing milk cans (Jamieson and Chen, 1944) and for general use in the dairy barn (Frayer, 1943). Mallmann and his coworkers (1946*a*) used a Quat effectively for destroying bacteria in milking machine equipment and Hucker (1947) recommended the use of the germicides in combination with nonionic synthetic detergents for the same purpose. A Quat solution was included in a program, along with chlorine disinfectants, in the control of *Streptococcus mastitis* in dairy cattle (Hendershott, 1945).

The Quats have been used in sanitation programs of other food processing plants such as (1) in the pickling industry (Bernstein and Epstein, 1948); (2) in the sterilization of dirty poultry eggs (Penniston and Hedrick, 1944); (3) in the handling of sea foods (Tressler, 1947); (4) in the bottling of beverages (Epstein, 1948); and in the brewing industries (Lehn and Vignolo, 1946). Quats have also been used to control infections in fish of hatcheries (Rackley, 1946; Ordal and Rucker, 1947) and for the prevention

of certain protozoal and virus diseases in poultry (Bolin, Goldsby and Eveleth, 1947; Tilley and Anderson, 1947).

The introduction of public automatic clothes washing machines created early recognition for some means to disinfectant the units between use of one customer to that of another. While the large commercial laundries have adequate facilities for providing large volumes of hot water of sterilizing temperatures, the small automatic installations for public use usually have inadequate supplies of water approaching that required for the destruction of microorganisms that may be found in soiled clothing (Lawrence, 1950). Thus, early investigations on the possible use of a Quat in the final rinse water in the automatic washing machines indicated that these compounds can be relied upon to give a reasonable margin of safety where a destruction of microorganisms on the washed fabrics is desired (Lawrence, 1950). The use of Quats in the final rinse water of diaper washing has also been found effective in controlling "diaper rash" in infants (Benson *et al.*, 1947, 1949); and has also been recommended for sanitizing bathing suits (Wallace, 1940). Some of the compounds have been advocated for the sterilization of plant protective equipment (Allis-Chalmers Manf. Co., 1947).

*Medicinal and Hospital Disinfection.*—A review of the literature will reveal that Quats have been widely used as skin disinfectants (Lawrence, 1950, 1951). Soon after the introduction of Quats, Wetzel (1935) reported his studies in which one of the latter compounds was found to be superior to the conventional use of alcohol in the hand surgical scrub. Shortly thereafter others described the successful use of Quat solutions for the treatment of various gynecological conditions and for the sterilization of surgical instruments (Schmidt, 1935; Caesar, 1935; Seeman, 1936; Rodecurt, 1934).

The use of chemical disinfectants with known germicidal activity in laboratory test procedures, has resulted in many recommendations for the use of the same compounds, with little proof as to their activity, in surgical procedures. Certain substituted higher complexes of phenolic derivatives have been adopted in the past few years as a substitute for the conventional Tincture of Green Soap in the surgical scrub. The low bacterial population of the normal skin, following the use of hexachlorophene (G-11)\* in various types of soaps, led to the introduction of this form of cleansing of the hands and arms in the presurgical scrub and for the preparation of the operative sites of patients who were to undergo surgery (Nungester *et al.*, 1949; Fahlberg *et al.*, 1948; etc.). However, since it was subsequently shown that the activity

\*Sindar Corporation brand of 2,2'-dihydroxy-3,5,6,3',5',6'-hexachlorodiphenylmethane.

of hexachlorophene was mainly one of a bacteriostatic nature, alcohol or an alcohol-acetone solution of a Quat was recommended in the final step of sterilization of the skin following the hexachlorophene-soap scrub (Seastone *et al*, 1949; Lawrence, 1952). Aqueous solutions and tinctures of Quats have been widely used in other services of hospitals and in the practice of dentistry for the sterilization of instruments and other appliances (Lawrence, 1950).

Quats have been used to an advantage by some workers as preservatives for ophthalmic solutions (Hind and Goyan, 1947; Chaimov, 1946) and also for increasing the penetrability of certain ophthalmic drugs into ocular fluid and tissues (O'Brien and Swan, 1941, 1942; Albough and Brady, 1946; Bellows and Guttman, 1943). Although the Quats have been extensively used as preservatives for various drug formulations, one must be guided in their use by information concerning the compatibility of the compounds with each specific agent or drug that is present in the formula.

Quats have been used in aerosols in which the compounds were found to increase the penetrating action of antibiotics and sulfonamides. In the combination with the former reagents the Quats were used to decrease the surface tension and accordingly increase the penetrability of the antibiotics in conditions of chronic bronchitis, bronchiectasis and certain pulmonary conditions (Grace and Bryson, 1947, 1948). Quats have also been combined with glycol aerosols in which the combination of the two forms of antimicrobial agents appeared to be more effective than the separate components in the formulation (Fulton *et al*, 1948).

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## 24

# PINE OIL DISINFECTANTS

## INTRODUCTION

PINE oil disinfectants possess germicidal properties which make them well suited for most disinfecting processes. They are effective against pathogenic microorganisms which cause communicable diseases, which is the principal function of this class of germicides. In addition they possess cleansing and penetrating properties, and also have a pleasant odor.

Recognition of the disinfecting value of pine oil emulsions was first emphasized by Stevenson (1915) of the United States Public Health Service, who not only advocated their use as general disinfectants, but specified the ingredients suitable for the purpose. This became known as the Hygienic Laboratory formula, which is the basis of pine oil disinfectants now employed. Although Stevenson specified rosin soap as the emulsifying agent, certain other soaps and sulphonated oils are also used for this purpose. Certain other variations, such as the quality of the pine oils used, methods of manufacture, etc., have been employed by different manufacturers.

A satisfactory pine oil disinfectant consists of a carefully balanced blend of the proper grade of pine oil and the proper grade and amount of saponifying or emulsifying material. Such disinfectants have phenol coefficients ranging, usually, from 5 to 6 or even higher, according to the quality and concentration of the active ingredient and the emulsifying agent. The nature and concentration of the saponifying agent has a direct effect on the germicidal activity of the pine oil emulsion, and the range of the phenol coefficient can be raised or lowered accordingly, even though the bactericidal property depends entirely on the quality and content of pine oil. Such emulsions of pine oil are effective in killing, on inanimate objects, those pathogenic bacteria which cause com-

communicable diseases, when dilutions 20 times the phenol coefficient are employed.

### SOURCE OF PINE OIL

Pine oil itself is a volatile oil which varies in color from almost colorless or light amber in the best grades to a much darker shade in the cheaper grades. It is obtained from waste pine wood, that is the stumps, dead trees, and fallen timber left in earlier operations throughout the pine lands of the southeastern states. The resinous heart of this wood becomes exposed to the weather as the outer layers soften, decay, and slough off. Through the natural processes of hydrolysis and oxidation ("weathering"), a change takes place in the oleoresins, and heavy oils not found in the unaged pine wood are formed. From these oils the pine oil of commerce is obtained by subjecting the wood to destructive distillation, or by extracting with superheated steam, or by steam followed by solvent.

### EXTRACTION OF THE OIL

In the destructive distillation method the wood is packed in coarse lengths in an iron retort and heat applied to drive off the volatile turpentine and oil. Since the end point of this process is charcoal, the heat is applied slowly until the volatile substances have been drawn off, then the heat is raised to carbonize the remaining wood. The fraction of the distillate, which is a mixture of turpentine and light pine oils, is then further fractionated and the pine oil recovered.

In the steam distillation process or the steam-solvent distillation process the wood which is to be extracted is broken down by chipping and shredding so that the steam and solvent may penetrate more readily. The shredded wood is packed in large retorts and subjected to the action of superheated steam. The turpentine and light oils pass off to be purified later while the rosin and heavy oils remain behind. If the process is to include the additional step of solvent extraction, the retort is allowed to cool, a hydrocarbon solvent added, and extraction with heat carried on until the rosin has been removed. This solvent extract is then drawn off and fractionated for rosin and pine oil and recovery of the solvent. The steam distillate of either process is fractionated into pine oil and turpentine.

### CHEMISTRY OF PINE OILS

Pine oil as originally defined is that fraction of distillate which either the destructive distillation or steam distillation of pine oleoresins distills between 170° and 350°C. The turpentine

fraction is removed below 165°C and no rosin is distilled below the upper limit of 350°C. The crude oil as thus refined is a mixture of liquid terpenes, of which alpha terpineol is most abundant as shown by Teeple (1908). Other constituents found present but in lesser amounts, as reported by Schimmel and Co. (1910), are alpha and beta-pinene, camphene, 1-limonene, dipentene, cineol, fenchyl alcohol, camphor, borneol, and methyl chavicol.

Since the boiling points of these fractions vary from 175° to 350°C, the character of a pine oil varies with the distilling range of the particular oil. The National Formulary (1950) describes pine oil as a colorless to light amber oil having a pinaceous odor and specifies that it shall have a distillation range such that 95 per cent will distill between 200° to 225°C. This range is fixed to eliminate the less valuable portions of the crude oil with low boiling points and low germicidal powers, and also the high boiling portions which have no practical value in pine oil to be used as a disinfectant. The middle portion specified contains the compounds which are the more active germicides and are present in substantial proportions. These include the alcohols—alpha terpineol, dihydro-alpha terpineol—and the secondary alcohols—borneol and fenchyl alcohol—and camphor in small amount. Hence the definition: "Pine oil is a volatile oil composed chiefly of tertiary and secondary terpene alcohols obtained by extraction and fractionation or by steam distillation of the wood of *Pinus palustris* and any other species of *Pinus*."

The characteristics of the crude pine oil and its components and their particular properties are discussed by Hogg and Little (1935). They cite the earlier work of De (1930) regarding the germicidal value of the various components of crude pine oil, and reported terpene alcohols as having phenol coefficients of from 6 for alpha terpineol to 10.9 for borneol, while the terpene hydrocarbons were less than 1.0. Their results in general confirmed De's findings except for borneol, and both report camphor to be about the same in germicidal value as the alcohols. Hogg and Little also point out that the hydrocarbons of the crude pine oil, absent in the refined, not only are of no practical value as germicides, but are the most difficult to emulsify and that they also depress the tertiary alcohols. The secondary alcohols have satisfactory phenol coefficients and have good surface tension lowering qualities, but are more difficult to emulsify than the tertiary alcohol, alpha terpineol. Camphor shows good germicidal value and is easily emulsified, but rates low in surface tension reduction as compared with the alcohols. Alpha terpineol surpasses in all these qualities and is the most valuable constituent of pine oil in a pine oil emulsion disinfectant.



It is evident that the most desirable pine oil for the preparation of disinfectants possesses a high alpha terpineol content; this will be obtained in an oil with a distilling range within the limits of 195° and 230°C. Commercial pine oils are available in various grades from which the manufacturer may select according to the characteristics or quality desired in the finished product. The specifications for some of these oils are in Table 96.

TABLE 96

	#1	#2	#3	#4	#5
Boiling point at 15.6°C (60°F)	0.941	0.934	0.9432	0.9441	0.940
Refractive index at 20°C	1.4832	1.4820	1.4825	1.4825	1.482
Distillation range					
5%	212.3	202.0	214.2	213.3	210.
50%	215.6	212.0	217.4	216.5	—
95%	218.2	218.0	220.8	218.8	220.
Flash point — F	188	173	188	185	180
Polymerization residue—%	0.4	0.4	1.4	1.6	0.4
Total alcohols—%	—	—	89	86	84
Pounds per gallon—15.6°C	7.84	7.78	7.86	7.87	7.83

## FORMULATION OF PINE OIL DISINFECTANTS

Since pine oil is not soluble in water it is used in the emulsion form, with soap, sulphonated oil, or some other emulsifying agent being used to obtain good dispersion in the aqueous mixture. The commercial disinfectant is a mixture of a pine oil, the selected emulsifier and water, the proportions being varied to obtain the desired strength as measured by the phenol coefficient with due regard to cost.

The emulsifying agent most commonly used in its manufacture is rosin soap, and a variety of rosin soaps is available from which selection can be made to suit the particular circumstances of its intended use. Soaps other than rosin soaps can be used alone or in mixtures with good results. According to Hogg and Little, the soaps in order of their effectiveness are linoleates, oleates, laurates, palmitates, and stearates. Sulphonated oils are also suitable, sulphonated castor oil being especially useful, and often produces an emulsion with a phenol coefficient of from 8 to 9.

Rosin soap is the emulsifier used in the original Hygienic Laboratory formula, which contains 1000 parts by weight of steam-distilled pine oil, 400 parts of rosin, and 200 parts of a 25 per cent solution of sodium hydroxide. Rosin is dissolved in the pine oil by means of heat, then the caustic soda is added and the mixture heated until the saponification is completed. A clear yellow homogeneous solution is obtained which upon the addition of water gives a white milky emulsion.

The amount of oil is about 62.5 per cent, which compares closely with the minimum of 65 per cent by volume specified by the National Formulary (1950) for Pine Oil Emulsion Concentrate and 60 per cent by weight specified in the U. S. Chamber of Commerce Commercial Standard for Pine Oil Disinfectant (1938). The water content is slightly in excess of the 10 per cent maximum set in the two above formulas.

The Hygienic Laboratory formula gives a product which has a *Salmonella typhosa* phenol coefficient of 3.5 to 4.5, while within the framework of the other two formulas disinfectants can be made with a range of phenol coefficients from 5 to 8 or 9. The usual or common type of pine oil disinfectant has a phenol coefficient between 5 and 6. The higher coefficients are obtained either by the use of a greater percentage of pine oil, the use of pine oil of a more carefully selected boiling range, the use of better emulsifiers, the use of less water, or by more skillful balancing of all of these factors. Although the better grades contain 60 per cent or more of pine oil, there are uses for types containing lower percentages with less germicidal activity. These have a phenol coefficient of 2 to 3 and are used where the deodorizing and cleansing properties of the pine oil emulsion are desired rather than disinfecting action.

## GERMICIDAL ACTIVITY OF PINE OIL DISINFECTANTS

Stevenson (1915) recommended the use of Hygienic Laboratory pine oil disinfectant in dilutions up to 1 to 500, and indicated that this germicide could be employed in place of other disinfectants. Two years later, however, McCoy, Stimson, and Hasseltine (1917) reported that this disinfectant was not effective against *Micrococcus pyogenes* var. *aureus* and therefore should not be used for the purpose of killing pyogenic micrococci. This was confirmed by Walters (1917) and later by Shippen and Griffin (1923), and others. It was generally agreed that pine oil disinfectants were suitable for use against non-sporing pathogens other than the pyogenic micrococci, and that their use should be limited accordingly. A dilution of 1 to 100 was recommended for general use as a disinfectant where the destruction of micrococci was not desired.

Because of the inability of pine oil emulsions to kill *M. pyogenes* var. *aureus*, it became apparent that this disinfectant could not be used in the place of other germicides for general purposes as at first recommended. Since under practical conditions of use pine oil disinfectants were known to be effective against the microorganisms causing enteric diseases, their germicidal activity against other

pathogens became of interest. Based on the results obtained by Eddish (1941) and others, the germicidal activity of Hygienic laboratory pine oil disinfectant is shown in Table 97.

TABLE 97

Method: Modified Rideal-Walker	
Temperature of Medication: 20° C.	
Proportion of Culture to Disinfectant: 0.5 ml to 5.0 ml	
Temperature of Incubation: 37° C.	
<i>Name of organism</i>	<i>Dilution necessary to kill in 5 minutes</i>
<i>Pasteurella pestis</i>	1-500
<i>Vibrio comma</i>	1-500
<i>Salmonella typhosa</i>	1-300
<i>Salmonella paratyphi</i>	1-250
<i>Salmonella schottmuelleri</i>	1-250
<i>Shigella dysenteriae</i>	1-200
<i>Escherichia coli</i>	1-300
<i>Salmonella enteritidis</i>	1-200
<i>Proteus vulgaris</i>	1-200
<i>Streptococcus pyogenes</i>	1-200
<i>Corynebacterium diphtheriae</i>	1-100
<i>Pseudomonas aeruginosa</i>	1-70
<i>Streptococcus</i> (non-hemolytic type)	1-100
<i>Diplococcus pneumoniae</i>	1-100
<i>Streptococcus mitis</i>	1-40
<i>Mycobacterium tuberculosis</i> (animal test)	1% kills within 30 minutes
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	does not kill

The ability of pine oil emulsions to kill the organisms of communicable diseases makes them well suited for use on the floors of public institutions and buildings. It has been well established that streptococci are commonly found on the floors of homes, schools, and public buildings, in the dust which accumulates with daily use, and the coliform organisms are often abundantly distributed in neglected rest rooms, toilets, etc. Pine oil disinfectants are as effective as other types of disinfectants for their removal, as has been shown by Walter and Hucker (1942) in a study of the pathogenic organisms found in public places. They found *E. coli* present in the toilets, lavatories, and rest rooms and betahemolytic streptococcus was found in the floor dust and dirt of hotels, school rooms, and other public buildings. Although *E. coli* is in general not to be classified as a pathogen its presence points to the possibility that pathogenic intestinal organisms likewise may be present.

Using cultures of *E. coli* and a streptococcus which they had isolated from a school room floor, Walter and Hucker carried out a series of tests to determine the effect of certain disinfectants on



these organisms when present on floors. Liquid cultures of the two organisms were spread over test strips of varnished oak flooring, and after the inoculum had dried various amounts of several disinfectants, among which was a pine oil disinfectant, were applied over the same area. After at least 1 minute of contact the boards were swabbed, the swabs rinsed in sterile water, and the dilution waters plated in media suited for the growth of each type of organism. It was found in the case of *E. coli* that a 1 to 90 dilution of pine oil disinfectant reduced the numbers from approximately 50,000 to 2800 with the use of a 0.3 ml of the disinfectant, and with the use of 0.6 ml the numbers were reduced to 10, whereas the streptococcus having an original count of over 50,000 was reduced to less than 10,000 by 0.1 ml, to 415 by 0.4 ml, and to 0 by 0.8 ml. These results were summarized as follows: "Laboratory experiments have shown that cresylic acid, coal tar, pine oil, and phenol disinfectants are effective in destroying most of the streptococci and coliform organisms inoculated onto oak flooring when a volume sufficient to thoroughly wet the surface is applied. No one disinfectant has been found to be consistently more effective than another in this test."

### EFFECT OF ORGANIC MATTER ON GERMICIDAL ACTIVITY

On the basis of the accepted rule that the proper dilution of a disinfectant for general use is 20 times the phenol coefficient, or the equivalent of a 5 per cent phenol solution, a dilution of 1 to 80 or 1 to 100 is generally recommended for pine oil disinfectants. That organic matter diminishes the germicidal activity of pine oil and other types of disinfectants is well known, according to the amount and kind of organic matter present. In a series of tests made by the writer using 3 per cent of organic matter, a mixture of 2 parts peptone and 1 part gelatin, the loss in phenol coefficient value varied from 8 to 25 per cent. This mixture of organic matter is that proposed by Anderson and McClintic (1912).

In a study of the effect of organic matter on the germicidal activity of disinfectants, Klarmann and Wright (1944) used as their organic matter 10 per cent horse serum and also 10 per cent of citrated horse blood. They found that a pine oil disinfectant which showed a phenol coefficient of 2.5 against *S. typhosa* in the absence of organic matter, showed a coefficient of 2.2 in the presence of 10 per cent of horse serum, and 2.7 per cent in the presence of 10 per cent of blood.

## STABILITY

Pine oil disinfectants are stable under conditions of storage. Tests by different investigators have shown that little or no change occurs over a prolonged period of time. For example, Hygienic laboratory pine oil disinfectant having an initial phenol coefficient of 3.88 decreased to 3.66 after 1 year at room temperature. Another formulation showed a decrease from phenol coefficient 5.5 to 5.2 after storage for 1 year. These differences are insignificant and, in fact, are negligible when the limitations of the test are considered.

## TOXICITY

Pine oil emulsions are non-toxic, non-irritating, and safe under ordinary conditions of use. Even 1 to 20 dilution of Hygienic laboratory pine oil disinfectant when injected subcutaneously and intraperitoneally into guinea pigs caused no ill effects. There was no evidence of caustic action, no physiologic reactions of any kind, and no fever. Also there is little or no irritation to the human skin following prolonged contact with the dilutions used in practice, that is, dilutions 20 times the phenol coefficient. In such concentrations, pine oil disinfectants kill pathogenic microorganisms which cause communicable disease and conform to the usual requirements of a disinfectant for use on inanimate objects.

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## 25

# OXIDIZING COMPOUNDS

## INTRODUCTION

THIS discussion is limited to products high in oxygen content or that liberate oxygen in their use. Oxidizing agents as disinfectants are dependent upon the reactivity of oxygen for their effectiveness. While particularly effective against anaerobic bacteria, they inhibit or kill many of the aerobic types. The extreme reactivity of nascent oxygen acts in a number of ways: it may react with products necessary for the cell's metabolism, depriving them of proper balance; it may react directly with the cell structure; or it may speed up metabolism to the detriment of the cell's growth. The extreme reactivity of the oxidizing products limits their use to special cases where reactive organic matter is absent or in low concentration.

## CHLORINE DIOXIDE

Chlorine dioxide ( $\text{ClO}_2$ ) is a powerful oxidizing agent. It is produced in the form of a gas by adding sulfuric acid to sodium chlorite and generators are available to produce the gas at the point of usage. Chlorine dioxide is bactericidal to common water-borne pathogens; in addition to its bactericidal effect in water, it has been shown to reduce taste and odors, making drinking water more palatable. It is particularly effective in removing tastes derived from algae and phenols in the water (Ridenour and Armbruster, 1949). It exhibits increased bactericidal efficiency with the increase in pH, the opposite being true of free chlorine. This difference is probably due to differences in hydrolysis of chlorine and chlorine dioxide. Chlorine hydrolyzes in water to form hypochlorous acid and hydrochloric acid. Alkaline hypochlorites are formed which reduce the hypochlorous acid content. Chlorine dioxide hydrolyzes only slightly and therefore indicates its use in alkaline waters as well as plant effluents previously softened or water-treated to pre-



vent corrosion. Since chlorine dioxide is more expensive than chlorine, the two are usually employed together in water treatment where chlorine dioxide use is indicated. Chlorine is used in the first stages and reduces the amount of chlorine dioxide consumed in the process.

Chlorine dioxide is a more effective sporicide than chlorine (Ridenour, Ingols, and Armbruster 1949). This is probably due to the fact that it has five reactive electrons in contrast to chlorine's one. It is used to control the after-growth of spores in water treatment plants and distribution systems. The lack of reactivity of chlorine dioxide with ammonia results in better bactericidal properties in waters containing ammonia.

Tests indicate that both chlorine and chlorine dioxide, or mixtures of the two in concentrations used in water treatment, will inactivate the Lansing strain of poliomyelitis (Ridenour and Ingols, 1946).

## HYDROGEN PEROXIDE

Hydrogen Peroxide ( $H_2O_2$ ) is usually employed in a solution containing 3 per cent by weight of hydrogen peroxide. It is used not only for topical application for cuts, scratches, and minor wounds, but also as a stain remover and mild bleaching agent. Its effective bleaching action on human hair is widely known. About 10 volumes of oxygen are liberated in the above solution. Hydrogen peroxide is available also in very concentrated solutions, as much as 30 per cent or more. Its effectiveness is due to the fact that it liberates nascent oxygen. Discovered in 1818 by Thénard, hydrogen peroxide has since been rather thoroughly investigated. It has a broad spectrum of bactericidal power and is sometimes used in the treatment of wounds where both mechanical agitation and flotation are needed. As a disinfectant, it is used entirely in special applications, since its instability and inactivation by organic matter reduces the amount of oxygen available for disinfection. Since it decomposes into water and oxygen, hydrogen peroxide is used where no taste, odor, or toxic residues may be permitted. It is particularly useful in the treatment of anaerobic infections.

Dittmar, Baldwin and Miller (1930) report that the bactericidal action of hydrogen peroxide is increased greatly by the presence of ferric and cupric ions which seem to act as a catalyst in the oxidation of bacteria. Tripp (1946) has shown that sufficient hydrogen peroxide can be introduced into the peritoneal cavity of guinea pigs to prevent anaerobic peritonitis under experimental conditions. It has been used effectively following operation for appendicitis.

Hydrogen peroxide is the basis of a number of dental preparations, and is used as an antiseptic and cleansing preparation for artificial teeth and dentures. Pyogens may be destroyed by adding hydrogen peroxide and bringing the solution to a boil.

### GLYCERITE OF PEROXIDE

Glycerite of peroxide (Thenardol) is a preparation containing urea peroxide, anhydrous glycerol, and 8-hydroxyquinoline. It is used in oral preparations since it is non-toxic and non-selective against bacteria. Its application provides the oral mucosa with a thin film which holds the antiseptic in close contact with the tissues. Since it decomposes at a slower rate than hydrogen peroxide-water mixtures, it provides oxygen over a longer period of time. The preparation may be stored without decomposition if kept free of moisture.

### ZINC PEROXIDE

A thick paste of zinc peroxide prepared in sterile water is capable of giving off a steady flow of oxygen. It has been found to destroy anaerobic and microaerophilic organisms and to inhibit a wide variety of aerobic bacteria (Meleney, 1952). It is usually applied as a dressing and kept wet, and after decomposition to yield oxygen, only bland zinc oxide and zinc hydroxide remain. Zinc peroxide dressings promote rapid healing of wounds and are easy to remove since the paste does not stick to the tissue. It is an excellent detoxifying agent against almost every kind of bacterial toxin.

Zinc peroxide may also be made into an ointment using a water solution of polyethylene glycol and is sometimes mixed with calcium peroxide. Zinc peroxide paste in conjunction with sulfonamide treatment is effective in the treatment of gas gangrene infection. While the sulfonamide inhibits the progress of the gangrene, nascent oxygen is required to destroy the anaerobic bacilli (Gwinn, 1944).

### OZONE

Ozone has been used extensively in water purification, particularly in England, for over 50 years, but its use for this purpose is rather expensive compared to chlorine or chlorine dioxide. Its use as an air deodorizer is growing rapidly with the development of new, less expensive ozone-producing lamps. There has been some contention that ozone is too irritating for use in aerial disinfection;

however, it has been shown that ozone produced by the new lamps is less irritating than that produced by previous methods.

Ingram and Haines (1949) studied the effects of ozone in the disinfection of water and its effects on various bacteria. There was a considerable variation in its effectiveness against different bacteria, since it is adsorbed by organic matter. Ozone has very limited commercial uses as a disinfectant; it is employed in treating water for the bottling trade. Recent studies have shown it to be an effective cysticide, killing *Endamoeba histolytica* in water.

### POTASSIUM PERMANGANATE

Potassium permanganate is a very powerful oxidizing agent and was used extensively before the advent of hypochlorites as a drinking water disinfectant. It is still used to some extent for this purpose and also for washing fruits and vegetables in parts of the world where it is less expensive or more available than the hypochlorites. It is usually employed in a 1 to 5 per cent solution for disinfection, but has the disadvantage of staining surfaces where it is employed. The solutions are very deep pink to purple in color, and on decomposition black manganese dioxide is produced. The zinc salt has been used for an antiseptic and astringent in dilutions of 0.1 to 0.3 per cent as mouth washes, gargles, and douches.

Panja and Ghosh (1943) demonstrated that a 1 to 5000 solution of potassium permanganate controlled contamination of *Vibrio cholerae*, *Salmonella typhosa*, etc. on fruits and vegetables. Potassium permanganate-treated vibrios can be used as a vaccine to produce anti-bodies. Sulfuric acid combined with potassium permanganate is an effective cleaning and disinfecting agent for glassware contaminated with tubercle bacilli (Goldie, 1947).

### SODIUM PERBORATE

Sodium perborate has been used for some years as an ingredient in dental preparations. In solutions of 1 to 3 per cent, it reduces the oral flora through the action of liberated oxygen. It is a rather strong alkali and hence should be used with care in most preparations. Like hydrogen peroxide, it is antiseptic, and also has considerable deodorizing properties.

### SODIUM DICHROMATE

Sodium dichromate is a powerful oxidizing agent, but its use as a disinfectant is limited due to its corrosive action. The familiar



laboratory glassware cleanser of sulfuric acid-sodium dichromate is a powerful disinfectant.

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## 26

# CHEMICAL DISINFECTION OF SURGICAL INSTRUMENTS

### INTRODUCTION

AS APPLIED to instrument germicides the term "disinfection" means the destruction of all vegetative microbial forms; whereas "sterilization" indicates that fungal and bacterial spores are also destroyed. Tubercle bacilli and viruses require special consideration; they are not included in "disinfection" as this term is ordinarily used.

Contaminated surgical and dental instruments can be disinfected, and even sterilized, by immersion in chemical solutions. The idea that this could be done is as old as aseptic surgery itself. In the 1870's Lord Lister, although interested primarily in chemical asepsis of the operative field, adopted the practice of immersing his instruments before use in aqueous 1 to 40 carbolic acid (Meleney, 1948) or the less corrosive mercuric cyanide (Brewer, 1939). A few years later the noted Berlin surgeon Ernst von Bergmann (1882) published a report on the aseptic treatment of wounds in which he gave a prominent role to preoperative disinfection of instruments in bichloride of mercury. Von Bergmann's recommendation was widely accepted and from that time on the use of instrument germicides has persisted.

As knowledge of surgical bacteriology and disinfection accumulated during the succeeding years it became evident, however, that the chemical method had serious shortcomings. Heating instruments in boiling water became a common practice in the late nineteenth century and still is. Eventually the search for more efficient ways to destroy bacteria with heat led to the perfection of autoclave sterilization, which is the safest and quickest method for preparing most types of surgical materials. Unfortunately,

however, both autoclaving and boiling in water damage delicate cutting edge instruments, cement mountings of optical instruments, nonboilable gut and certain kinds of rubber and plastic goods. For these special purposes chemical disinfection is the method of choice. And because no bulky and expensive equipment is needed, it continues to be used more or less routinely for small instruments in many operating rooms, accident dispensaries and practitioners' offices (Ecker and Smith, 1937; Spaulding, 1939). Instrument germicides have an even greater area of usefulness in the dental profession. According to Brewer's survey (1939) 71 per cent of dentists advocated the chemical method for some types of instruments.

The safety, and therefore the practicability, of chemical disinfection depends very much upon the factor of time. If this method can free instruments of harmful microorganisms in 10 minutes or less, its popularity is justified. On the other hand, it is not only impractical but also potentially dangerous if many hours are required.

Nowhere else in the field of disinfection is there so much confusion and contradiction as exists around the question: How good is chemical disinfection of instruments? There are several reasons for this. Manufacturers sometimes recommend a product without *pertinent* data to support claims that upon investigation turn out to be entirely unjustified. Particularly dangerous are erroneous statements which give the user the impression that a relatively high dilution of a mercurial or quaternary ammonium compound will rapidly destroy spores on instruments. Sometimes this thought is conveyed by the phrase "spore-forming bacteria."

The harm done by overzealous promotion of proprietary germicides is in no small way responsible for the somewhat reactionary attitude of well-informed surgeons. Walter (1948), for example, says "Chemical disinfection of instruments should be limited to those which can be prepared in no other way, because it is time-consuming and unreliable unless carefully supervised and conscientiously performed." More recently (1953) he took an even stronger stand by stating that ". . . no known germicide at present actually sterilizes instruments." Although there are reliable data to show that certain disinfectants actually do sterilize instruments, there is, indeed, good reason for opinions such as these.

Furthermore, the present state of confusion is not limited to proprietary products. Insufficient attention has been paid to the development of adequate bacteriologic methods for the proper evaluation of instrument germicides. And the complexities of the problem are not generally understood. The numerous germicidal solutions now available vary tremendously in germicidal activity.



as do also the conditions under which they are used. A lightly contaminated scalpel carefully cleansed before exposure to germicide is much more easily disinfected than a hinged instrument covered with dried pus containing spores. Many available germicides could promptly disinfect the scalpel, but very few of them would sterilize the hinged instrument.

Laboratory tests can be designed to produce almost any desired result. It is a simple matter, for example, to simulate instruments which are lightly contaminated with spore-free bacteria and relatively free of extraneous organic matter. What little disinfectant action is needed in this instance will occur rapidly with the result that an impressively short disinfection time is obtained. On the other hand, conditions representing extreme contamination can be established by exposing hinged instruments to germicide while they are coated with dried pus or blood containing large numbers of spores. Under such severe conditions as these only the strongest germicides can sterilize at all, and these may require several hours to do it. Thus, a "disinfection time" of 10 minutes has little meaning unless the test conditions are specified; in fact, a test value of 10 minutes may conceivably be a poorer result than one of 3 hours.

In these introductory paragraphs an attempt has been made to explain why confusion exists. Nevertheless the real value of instrument disinfection can be determined providing those factors which influence the dynamics of the process are recognized and their effects measured. Because adequate bacteriological methods are available or are being devised to do this, there is good reason to believe that clarification of the problem is near.

## PRINCIPLES

The general dynamics of instrument disinfection are the same as for other inanimate objects in that the rate of microbial destruction is directly proportional to the number of organisms in the environment and to their resistance, but inversely proportional to the concentration of germicide. But unlike other inanimate objects, surgical materials are brought into intimate contact with human tissue. Because the safety of the patient is the primary consideration, all factors which significantly affect the disinfection time should be recognized and examined.

Of greatest significance in this respect are the type of instrument to be disinfected and the extent to which prior mechanical cleansing has been carried out. Hinged instruments with protected devices constitute a far more difficult object to disinfectant than flat and detachable knife blades. And thorough washing with soap

and water followed by a water rinse can actually remove all microorganisms from a flat instrument even before contact with a germicide. Thus the conditions of use must be clearly defined.

### FACTORS AFFECTING DISINFECTION TIME

**NATURE OF THE CONTAMINATING MICROORGANISM.**—Surgical instruments may become contaminated with bacteria, fungi, animal parasites and viruses. Although it can be expected that protozoa and helminthic eggs may at times be left on instruments, this is probably an uncommon occurrence; at any rate very little attention has been paid to the animal parasites as a group.

**Bacterial Spores.**—The most important microbial factor to affect the disinfection time is the presence or absence of bacterial spores. These highly resistant forms may survive for days in a chemical solution which promptly destroys the vegetative cells of the same species. *Clostridium tetani* is a good example. Knife blades contaminated with large numbers of nonsporulating bacilli can be disinfected in about 10 minutes by aqueous 1 to 1000 benzalkonium chloride, whereas they will not be sterilized under the same conditions in 3 days if only a few tetanus spores are present.

This deficiency would be of no great consequence if spores only rarely contaminated surgical instruments. But Brewer found (1939) that spore-forming anaerobes were present on 12 per cent of the knife blades used during routine operations. Eight per cent of these had been used for skin incision. Roberts and coworkers (1933) recovered *Clostridium welchii* from 8.5 per cent of knives employed for cutting skin in supposedly clean areas. These studies did not determine the actual presence of spore forms, a question Spaulding (1939) attempted to answer by making spore counts of blades which had been dipped in specimens of surgical pus. The number of spores per blade ranged from 0 to 15, with an average of 2. Thus, it appears that instruments used in routine infected cases are commonly contaminated with a small number of spores. Dirty wound infections, however, should not be considered as routine cases. A limited number of observations in the writer's laboratory suggest that blades so contaminated may carry as many as 100 spores. Another situation where spore contamination may be high is in large bowel surgery. Fecal specimens collected at the operating table often contain viable spores at the rate of 10,000 or more per gram (Spaulding, 1953).

Chemical solutions are available which have the ability to sterilize spore-bearing knife blades. Most of them are too corrosive for metal to be of any practical use as instrument germicides. A

strong hypochlorite solution such as "Chlorox" is an example. But alcohol-formalin mixtures can be prepared which are noncorrosive and truly sporicidal in that they destroy large numbers of spores on dried bloody knife blades within  $\frac{1}{2}$  to 6 hours, depending upon the resistance of the spores used. Even with these highly active preparations, the difference in the time for disinfection (vegetative forms only) and for sterilization (spores) is a few minutes on the one hand and several hours on the other.

*Tubercle Bacilli*.—Although the risk of spreading tuberculosis by the use of contaminated endoscopic and operative instruments has been recognized for a long time, this danger has by no means been overcome. Objects which do not withstand heat have to be treated with a chemical disinfectant, usually without knowledge of its efficacy. Until very recent times there was no good method for growing small inocula of tubercle bacilli in a fluid medium or obtaining dispersed growth. Consequently experiments with instruments and other solid objects were not only technically difficult but the results were generally unreliable. With the development of the tween-albumin type of culture medium, however, it should be possible to carry out satisfactory evaluation tests with real or simulated instruments. Although there is no information as to the frequency with which *Mycobacterium tuberculosis* contaminates dirty and mechanically cleansed instruments, the very serious nature of the disease it produces demands that the efficacy of chemical germicides in the tuberculo-disinfection of instruments be established as soon as possible.

The high level of resistance of acidfast bacilli to certain chemical agents is well recognized. In fact, it is by virtue of this tolerance that sputum specimens are freed of other types of living vegetative bacteria in the routine preparation of "t.b." cultures. Any organism which withstands 30 minutes' exposure to 4 per cent sodium hydroxide can be expected to present a serious problem in instrument disinfection.

Published data on the resistance of tubercle bacilli are highly conflicting as can be illustrated by the cresols. McCulloch (1945) summarized the data of Klarmann and his group which indicates that *Myco. tuberculosis* is killed by the same concentrations that destroy other non-sporulating bacteria, and that 1 to 200 dilution of cresol is tuberculocidal in 10 minutes. On the other hand, Middlebrook and Freund (1952) state that a 1 to 50 concentration requires 12 hours. The latter authors also make the comment that tubercle bacilli are extremely susceptible to ionic detergents, a point which is contradicted by the findings of Smith (1951). The reasons for these discrepancies are the technical difficulties associated with



culturing virulent tubercle bacilli by older methods as well as the failure to realize that very small amounts of germicide may produce tuberculostasis.

The tubercle bacillus is definitely more resistant than other vegetative microorganisms to acids, alkalies, quaternary ammonium compounds and probably all aqueous germicides. On the other hand, this insusceptibility is not as great as that of spores. It is now recognized that the usual acid or alkali treatment of sputum has a significant destructive action on acidfasts, and Smith (1947) reports cidal action by 50 to 70 per cent ethyl alcohol and by 30 to 80 per cent isopropyl alcohol in 1 to 2 minutes with his dried sputum smear technique. From these scattered observations it appears likely that the rapid destruction of tubercle bacilli on surgical instruments can be accomplished by certain types of germicide. But until more reliable information is available, acidfast organisms must be regarded as having a chemical resistance intermediate between that of vegetative bacteria and spores.

*Fungi*.—The vegetative forms are about as susceptible as bacteria to destruction by heat and chemicals. But, as Emmons pointed out (1933), resistance varies widely among species and among strains within species. Spore forms are produced by all molds and exist as several different types. The conidia of *Trichophyton mentagrophytes* are somewhat more resistant to phenol than are bacteria, being destroyed by 1 to 45 dilution in 10 minutes at 20°C, but not by 1 to 60 (Emmons, 1945). It is likely that the presence of chlamydospores, which are thick-walled and comparatively resistant to environmental conditions, markedly prolongs the disinfection time of instruments. The relevant experiments have not been performed. When they are, the technique of Emmons (1945) should be consulted, and the resistance to heat or a standard chemical compound determined.

*Viruses*.—There is almost no definitive information on the transmission of viral infections by contaminated instruments. An extremely important exception, however, is that of serum hepatitis (SH), also known as homologous serum jaundice. Improperly handled hypodermic needles and syringes constitute the major route of transmission of this common and serious disease. In view of the recent report (Drake *et al.* 1952) that 1 ml of blood may contain as many as 25,000 infectious units, it is imperative that extreme care be applied to the disinfection of these materials. Even the simple act of pricking the finger for a blood count becomes a potentially dangerous procedure. Since the related virus of infectious hepatitis is known to be present in blood and feces, it is likely that this too will eventually be found on instruments. Both of these

viruses resist 56°C for at least 30 minutes (Havens and Paul 1952). And SH virus remains viable for many months in serum containing 0.2 per cent tricresol (Neefe 1946). There is no knowledge as to the effectiveness of alcohol, formalin, quaternary ammonium compounds or any other germicide against SH virus, and certainly none with specific application to instruments. Considering the fact that human beings are the only susceptible hosts there seems to be no practicable way to study the problem.

TABLE 98.—EFFECT OF THE NUMBER OF BACTERIA UPON THE DISINFECTION TIME

Staphylococcus #209		Aqueous Formaldehyde	
		Dilution endpoint	
Inoculum count	Test procedure	Positive	Negative
$\times 10^8$	Free suspension (no blood)	1:20,000	1:30,000
$\times 10^6$	" " " "	1:40,000	1:50,000
$\times 10^4$	" " " "	1:50,000*	1:60,000
* Delayed (5 days) growth			
Staphylococcus #209		Benzalkonium chloride, aqueous 1:1000	
		Results	
Blade count	Test procedure	Positive	Negative
$2 \times 10^7$	Dried bloody knife blade	45 minutes	60 minutes
$5 \times 10^4$	" " " "	5 minutes	10 minutes
$5 \times 10^3$	" " " "	5 minutes	10 minutes
B. anthracis #33A		8% HCHO + 0.5% G-11 in 67% isopropanol	
		Boiling water	
Spore count	Test procedure	Positive	Negative
$\times 10^7$	Dried bloody knife blade	30 minutes	?
$\times 10^4$	" " " "	15 minutes	20 minutes
$\times 10^3$	" " " "	10 minutes	15 minutes

This unfortunate situation, however, does not exist with viruses which can be grown in the chick embryo or in small laboratory animals. Thus the method employed by McCulloch (1945) with fowl pox virus appears to be applicable to instrument disinfection. The fact that viral studies have not been carried out with instruments reflects the general lack of interest in viral disinfection of instruments and not its real importance.

NUMBER OF MICROORGANISMS.—It takes longer to disinfect or sterilize an instrument contaminated with a million bacteria than it does when only a hundred are present. This observation is, of course, in accord with the general principles of disinfection, but here it has a special significance. If instruments are still heavily contaminated at the time they are placed in germicide, they may be removed by the careless or impatient user before disinfection has occurred. It is important, then, to have some definitive data on this point. The figures in table 98, obtained in the writer's

laboratory, are presented to show that the effect of inoculum size is a significant one and essentially the same whether one is dealing with vegetable bacteria freely suspended in blood-free germicide or spores on a dried bloody knife blade, also that it applies equally well to bacteriostatic, bactericidal or sporicidal procedures.

**PRIOR CLEANSING OF INSTRUMENTS.**—Under practical conditions the number of contaminating microorganisms is largely a function of the extent to which the instruments have been cleansed prior to immersion in germicide. Any instrument which has been in contact with body fluids is coated with a layer of proteinaceous

TABLE 99. DISINFECTION TIME OF "CLEAN" AND BLOODY INSTRUMENTS

Germicide	Organism	Procedure:	Count per blade	Results	
		Dried knife blade		Positive	Negative
A	Staphylococcus #209	50% blood	$9 \times 10^6$	10 minutes	15 minutes
		No blood	$9 \times 10^4$		15 seconds*
B	Staphylococcus #209	50% blood	$9 \times 10^6$	10 minutes	15 minutes
		No blood	$9 \times 10^4$		15 seconds*
A	Group A streptococcus (O strain)	50% blood	7200	5 minutes	10 minutes
		No blood	10 to 20		15 seconds*
C	Staphylococcus #209	50% blood	$4.2 \times 10^6$	5 minutes	10 minutes
		No blood	$3.5 \times 10^4$		30 seconds*
C	E. coli #4932	50% blood	$4.5 \times 10^7$	10 minutes	15 minutes
		No blood	$4.5 \times 10^4$		30 seconds*
* Shortest test interval used					
C	B. anthracis-T48	Spores			
		50% blood	$2.3 \times 10^6$	1 hour	1½ hours
		No blood	$6 \times 10^3$	1 hour	1½ hours
Free suspension					
D	Cl. tetani-Hall	Spores			
		10% blood	$1.7 \times 10^6$	2 hours	3 hours
		No blood	$1.7 \times 10^6$	2 hours	3 hours
Germicide A Benzalkonium chloride, aqueous 1:1000					
" B—1% hexachlorophene in 8.75% isopropanol					
" C—8% formaldehyde and 0.5% hexachlorophene in 67% isopropanol					
" D—8% formaldehyde in 67% isopropanol					

material which coagulates and upon standing becomes a dry film. Often pieces of solid tissue are also present. Especially if drying has occurred, the microorganisms are contained within, and protected by, a more or less continuous mechanical barrier. Under experimental conditions instruments—even small flat ones—coated with organic matter can be made to carry enormous numbers of microorganisms, and it is conceivable that these extreme conditions are at times approached in actual practice.

Since many instrument germicides are protein coagulants, contact with the coating of organic material renders it even less penetrable, and the result is a markedly prolonged disinfection time. Whether it be wet or dry, the presence of extraneous organic matter



reduces the activity of all germicides, some more than others. Thus, prior cleansing is even more important when instruments are being prepared for chemical disinfection than it is for heat sterilization. Yet experience has shown that users can not always be depended upon to do a thorough job. Therefore an adequate set of evaluation tests must allow for conditions approximating those on bloody instruments which have been permitted to dry for several hours.

The importance of removing blood and other organic matter is easily established by laboratory tests. It is not necessary to go through the actual process of cleansing contaminated instruments; the results are erratic. Instead, the test objects are examined under two sets of conditions which are identical in every respect except that blood is omitted from one of them. When this is done the differences in disinfection time are very large (table 99). On the other hand, sporicidal times are influenced very little, due possibly to the fact that spores are destroyed only after long contact with germicide under the best of conditions.

**TYPE OF INSTRUMENT TO BE DISINFECTED.**—Flat instruments can be disinfected within a few minutes by most of the available germicidal solutions. Those with hinged joints and hidden crevices, however, are another matter. Despite the fact that evaluation tests are carried out on a single type of instrument and under controlled experimental conditions, the results still show considerable variation. Therefore it stands to reason that even more variation occurs in actual practice where all sorts of instruments are being handled together. Even with strong germicides such as aqueous and alcoholic formaldehyde solutions, the time required for disinfection varies all the way from a few seconds for a "clean" knife blade to 30 minutes or more for a dried bloody hinge.

Instruments with lens systems constitute a special problem. Strongly alkaline solutions affect the glass after prolonged or repeated exposure. The cement mountings of many telescopic instruments are not only damaged by heat but by organic solvents as well. From the germicidal standpoint the most effective germicide is an aqueous solution of formaldehyde followed by thorough rinsing and drying. But one of the cationic detergents may be substituted if a less irritating chemical is desired. The time required for chemical disinfection of telescopic instruments has never been determined under controlled conditions; when it is, prime consideration must be given *Myco. tuberculosis*.

The chemical disinfection of needles and syringes is slow and unreliable unless prior cleansing is thorough. Even if this is done, casual immersion in a chemical solution often results in failure due to retention of air pockets in the lumen. Needles, therefore, should

first be flushed with a cleanser such as Haemosol\* and then flushed with the germicidal solution before immersion. The same procedure is needed for syringes. If these precautions are carefully observed, bacteriological safety can be achieved. But they do not completely avoid the risk of serum hepatitis which is transmitted by the needle and syringe route. Because we know nothing about the susceptibility of this virus to instrument germicides, it is advisable to autoclave such materials or place them in boiling water for 15 to 30 minutes. Whenever chemical disinfection is employed, the procedure should be 30 minutes' exposure in a strong germicide such as an alcohol-formalin mixture.

**CONCENTRATION OF GERMICIDE.**—The relationship between germicidal concentration and the rate of microbial destruction is a well-established principle which has practicable significance in the use of instrument germicides. The proper concentration must be great enough to disinfect within a period of time that is convenient for the user. This can only be determined by means of relevant laboratory tests.

## METHODS

**GENERAL PRINCIPLES.**—Theoretically, the ideal way to evaluate a surgical germicide is to test instruments actually used in an operative procedure. But the conditions are so variable that none but the occasional attempt yields significant information. It is better, instead, to determine the type and extent of contamination on sample instruments, and then duplicate the desired conditions in controlled laboratory tests.

In support of this principle the published opinions of two well-known students of disinfection warrant reproduction. Walter (1948) in his excellent treatise on the aseptic treatment of wounds says, "Most laboratory tests are not susceptible of clinical interpretation because the conditions of the experiment are not similar to those found in clinical problems." That the situation need not exist was emphasized by Garrod (1935) as follows: "It is customary to deride laboratory conditions as being too far removed from those of practice to afford significant results; the truth is that practical conditions can be imitated closely and in almost every particular. The virtue of a test may in fact be held to depend largely on how far it achieves this, and the whole art of devising it consists in securing the necessary conditions without sacrifice of uniformity."

The imitation of practical conditions would include the use of representative types of surgical and dental materials. Unfortunately

\*Meinecke and Company, New York City.

ly, large objects such as transfer forceps and optical instruments are too awkward to handle on a large scale. Thus, one is essentially limited to knife blades, small cutting edge implements, small hinged instruments, needles and syringes. Most convenient to use are detachable knife blades.

Although the principle of trying to duplicate clinical conditions should be generally adhered to, this is sometimes inadequate. The number of microorganisms recovered from sample instruments is ordinarily less than 100. Tests carried out with so few organisms are likely to give irregular results. Furthermore, heavier contamination is desirable because it provides a margin of safety for the occasional situation where prior cleansing has been careless. As will be outlined later, an adequate evaluation procedure includes a variety of tests, each of which is intended to represent a particular situation encountered in practice.

Because tests on instruments themselves are somewhat laborious, time and effort can be saved by first setting up selected standard tests. Good use can be made, for example, of the broth dilution inhibition method and cidal techniques in which microorganisms are freely suspended in disinfectant solution. These serve as good screening tests in that a group of disinfectants compared by these less practical methods have in general the same relationship to each other as are later found to be the case with instrument tests. It should be noted, however, that the disinfection time values obtained with real or simulated instruments are generally much longer than those with the screening procedures (Tainter *et al.* 1944).

The types of microorganisms most commonly encountered on surgical and dental instruments should be employed for test purposes. These are staphylococci, coliform bacilli and enterococci, followed by various *Proteus* and *Pseudomonas* species. Hemolytic streptococci and pneumococci are commonly present on dental but not on surgical instruments and, because of their highly susceptible nature, need not be included. Diphtheroids and various non hemolytic streptococci are ubiquitous, but there seems to be no good reason for considering them as test organisms. On the other hand, *Myco. tuberculosis* should by all means be included even though there is no information as to the frequency with which it occurs on instruments.

The spore types most frequently recovered from used instruments are air contaminants belonging to the *Bacillus* group. Therefore, spore suspensions of a *B. subtilis*-like species or of *B. anthracis* are appropriate. Among the pathogenic clostridia, tetanus spores are the most resistant; and because they constitute a major risk from the use of germicides, this is the spore type of choice.



## INSTRUMENT TESTS

*Knife Blades.*—The method described by Spaulding (1939) is convenient and produces fairly reproducible results. Any type of situation encountered in the use of flat instruments can be approximated by varying the test conditions. For example, a blade dipped in a light broth suspension of bacteria resembles a cleansed instrument—but still provides a desirable margin of safety. Permitting this blade to dry before chemical treatment is an appropriate modification. In order to test the capacity of the germicide to disinfect instruments which are not only allowed to become dry after use but also placed in the solution without cleansing, one can prepare heavy suspensions of organisms in blood, serum or pus. Blades thus contaminated are then allowed to stand for several hours before chemical treatment.

A considerable amount of disinfectant is carried over to the recovery medium during subculture. Therefore, the blade is first rinsed in 10 ml of broth before transfer to a second 10 ml tube. Both tubes should be incubated for evidence of growth. Providing an efficient neutralizer is available, a 10 ml volume of broth is adequate. When none is available, however, larger volumes must be employed, the exact quantity of which is determined by preliminary testing under conditions identical with those of the intended test procedure except for the substitution of a 1 to 100 or 1 to 1000 dilution of the inoculum.

Tainter and coworkers (1944) used metal strips as simulated knife blades and a technique of drying which produced conditions closely approximating those of actual usage.

*Hinged Instruments.*—The use of whole instruments is expensive and generally impracticable because large volumes of disinfectant solution and culture broth are required (Post and Moor, 1942). Instead, the author has used the joint portion of discarded instruments. All is cut away except a one inch-long piece which includes the hinge itself. The type of joint varies from one instrument to another, as does the thickness of the implement. Nevertheless, reasonable uniformity can be obtained by matching similar pieces.

Simulated hinges can also be used successfully. The writer now employs pieces of stainless steel 1 inch long and  $\frac{3}{8}$  of an inch wide. They are paired so that one has on its side a pin which fits into a slit in the other piece. One of the pieces is bent so as to produce a shearing action when rubbed against the other. This simulated joint can be opened and closed. The two pieces are separated entirely when placed in recovery medium. Simulated hinged

oints have also been devised by Brewer (1945) and Lewison (1950).

*Hollow Needles and Syringes.*—The syringe and needle assembly is contaminated by drawing a blood-microorganism mixture up into the barrel and then expelling it. The assembly is then permitted to stand at room temperature for 2 hours in a sterile container. Since needles cannot be chemically disinfected if the cannula is full of clotted blood, a cleansing procedure must be carried out. This should be exactly the same with each assembly used in a single test. Before the needle is immersed in the germicide a sufficient volume of the solution is drawn up through the needle to fill the syringe barrel; it is then expelled into a larger test tube or small flask. The separated needle, barrel and plunger are then placed in separate large tubes of germicide where they remain for the duration of the test, at which time each of the three parts is transferred to separate tubes or flasks of recovery medium.

*Other Materials.*—Small dental and ophthalmological instruments can be handled much the same way as knife blades or hinged instruments. Catheters and optical instruments require special procedure which must be devised as the need arises.

## SPORE TESTS

The basic procedures are the same as for vegetative bacteria. The most important technical point is to make certain that one is actually dealing with spores. All too often published data purport to show that a low concentration of a halogen, phenolic or quaternary ammonium compound destroys spores. Notwithstanding the fact that this is a surprising result, these reports fail to mention spore counts or give any other indication of the resistance of the test suspension. Occasionally the term "*Cl. tetani*, a spore-bearing organism" is used rather than the word tetanus spores; whether such terminology be intended as a subterfuge or not, the inference in the mind of the uninitiated is the same. Reports of tests upon bacteria capable of producing spores should clearly state whether spores were present or not. If they were, the spore counts should be given. Furthermore, some indication of the resistance of those spores is highly desirable; this can be the survival time in boiling water or in 1N hydrochloric acid at room temperature.

Whenever spore tests are limited to one bacterial species, that one should be *Cl. tetani*. It is best, of course, to use several different types so as to encompass species variation. The writer has made a practice of using *Cl. tetani*, *B. anthracis* and *Cl. welchii* (*perfringens*) spores with the occasional inclusion of other clostri-

dia and unidentified, highly resistant air contaminants belonging to the *Bacillus* genus. The genus to which the spore belongs is relatively unimportant; but what is necessary is to count the number of cell elements in the inoculum capable of surviving 80°C for 15 minutes. This figure should be given in relation to the specific volume of germicide used regardless of whether it refers to an instrument or a free suspension.

Satisfactory suspensions can be prepared from agar growth or from washed centrifuged fluid cultures. These are stored at refrigerator temperature until an aliquot has been assayed for spore content and resistance to boiling water. The test suspension should not be heated prior to use since the presence of susceptible non-sporulating cells has no effect upon the results except to provide added organic matter.

### TUBERCLE BACILLI

There is no established procedure for instrument tuberculocidal testing. In as recent a report as that of Lawrence *et al.* (1952) tubercle bacilli are freely suspended in germicide without the addition of body protein even though the tests are stated as performed with instrument sterilization in mind. Earlier experiments by Lawrence and Grover (1951), however, did make use of a urine-gastric mucin mixture and of tuberculous sputum which after treatment were subjected to guinea pig inoculation for evidence of survival. One of the techniques employed by Smith and his group (1951) may well approximate the conditions existing on contaminated flat instruments. Naturally positive sputum was spread on a coverglass and allowed to dry. Following immersion in the test solution for the desired period of time the slide was transferred to weak hydroxide solution containing glass beads and the organic material removed by shaking. The writer has carried out a limited number of tests with the knife blade technique described earlier and using a tween-albumin broth as the recovery medium. Although the last-mentioned procedure is not entirely satisfactory, it warrants further investigation with special emphasis upon the need for using both animal inoculation and culture methods for the detection of survivors.

The selection of the test strain is important. Old stock strains labelled *Myco. tuberculosis* will sometimes be found to contain nothing but gram positive cells, acidfastness having been entirely lost. They are not only avirulent for the guinea pig but highly susceptible to chemical action. To a less degree the same thing can be said of nonpathogenic species such as *Myco. ranae*, *Myco. phlei*



and *Myco. smegmatis*. It is highly desirable, if not essential, to use a virulent human strain of *Myco. tuberculosis*. For the sake of conformity all laboratories should use the H37rv strain and check its virulence every 6 to 12 months by guinea pig inoculation.

## TYPES OF INSTRUMENT GERMICIDES

A large number of proprietary and nonproprietary germicidal solutions have been tested or employed for instrument disinfection. It would be fruitless and misleading to attempt a detailed review of their antimicrobial activity as reported in scientific publications and manufacturers' brochures. Frequently, obvious violations of experimental procedure nullify the interpretation that a bactericidal or sporicidal effect has occurred. It may be helpful, however, to comment in a general way upon the demonstrated or probable value of the classes of germicides most widely used for instrument disinfection at the present time.

### ORGANIC MERCURIALS

As a class they are devoid of sporicidal power and are relatively poor disinfectants, at least in the low concentrations usually employed (Brewer, 1939). Their extremely high level of bacteriostatic and fungistatic activity is useless in instrument disinfection. On the basis of data obtained by Lawrence and Grover (1951) one is safe in assuming that aqueous mercurials will not destroy tubercle bacilli in instrument tests containing much organic matter, although tinctures may have some value by virtue of their alcohol content. The meager observations reported up to this time offer no reliable indication of the viricidal properties of organic mercurials.

### PHENOLIC DERIVATIVES

Cresol-soap mixtures and "Lysol" (cresylic acid and ortho-hydroxydiphenyl) are good disinfectants even in the presence of extraneous organic matter. But they have an objectionable odor and irritate tissue. Several non-odorous derivatives are now available; in fairly high concentrations they show promise as instrument germicides.

Most of the phenolic compounds which have been subjected to instrument tests disinfect rapidly, but exhibit little or no sporicidal activity. As a class they are satisfactory fungicides. From the results of Smith (1951) and Lawrence and Grover (1951) one is tempted to predict that cresol, cresylic acid and p-tertiary phenol,

at least, will prove to be effectively tuberculocidal in instrument tests. The older phenolics are strikingly ineffectual against viruses; what the newer derivatives can do remains to be determined.

## CHLORINE

Inorganic chlorine compounds, although highly germicidal in the absence of extraneous protein, are too corrosive for instruments. When combined with certain organic compounds, particularly phenolic derivatives, chlorine is a more practical disinfectant. Generally speaking, however, these chloro-phenolics are unsuitable for contact with instruments. A notable exception is hexachlorophene (G-11). Although employed most often as an antiseptic, a 1 to 100 concentration has been commercially prepared in suitable form for instrument use. In tests using contaminated instruments it is satisfactorily bactericidal and fungicidal, but it is not sporicidal or tuberculocidal. Its virucidal activity is unknown.

## QUATERNARY AMMONIUM COMPOUNDS

The large number of chemicals in this class makes generalizations difficult. They retain their germicidal activity well in the presence of extraneous protein, and they are relatively nonirritating in the low concentrations generally employed. By selecting benzalkonium chloride as representative of the more highly germicidal ones, it is possible to state that they are good instrument disinfectants but poor sporicides. Data published earlier by Spaulding (1939) indicated that benzalkonium chloride destroyed spores in 18 hours on dirty knife blades. Subsequent experience, however, has not supported these findings. If an efficient neutralizer is added to the recovery medium, tetanus spores consistently survive 48 hours' exposure. In this connection the report of Klarmann and Wright (1950) on the purported sporicidal properties of another quaternary ammonium compound is well worth reading.

Lawrence (1950) states that mold spores are extremely resistant to practically all types of chemical agents, but then cites studies which indicate that 1 to 500 aqueous benzalkonium chloride destroys fungal spores in 5 minutes. From the pertinent data available it seems safe to assume that this and equally active quaternary ammonium compounds are satisfactory instrument fungicides.

Aqueous solutions probably have little value as tuberculocides (Smith, 1951; Lawrence and Grover, 1951). Since *Myco. tuberculosis* is inhibited by dilutions as high as  $1 \times 10^{-6}$  (Lawrence, 1950), it is essential that tuberculocidal tests be carefully controlled and

the results cautiously interpreted. The writer was recently prompted to test aqueous 1 to 1000 cetyltrimethylammonium bromide chloride because of a verbal report that it was rapidly cidal for tubercle bacilli. When plain tween-albumin broth was used as the recovery medium, no growth occurred in any of the tubes, even those representing the 5 minutes exposure period and the controls; whereas the simple expedient of adding 10 per cent blood to the recovery broth produced growth in all tubes including those inoculated with tubercle bacilli exposed to germicide for two days.

Lawrence, (1950), in reviewing the virucidal properties of surface tension depressants calls attention to the conflicting nature of the reports on benzalkonium chloride. Under exactly the same conditions some viruses are inactivated, others are not; and the same virus which was previously destroyed becomes resistant when the conditions are altered slightly. Apparently viruses differ in their susceptibility to chemicals in much the same way as the various species of bacteria and fungi. However great this variation may be, it appears unlikely at the present state of our knowledge that the quaternary ammonium compounds in a low concentration (1 to 1000) will prove to be instrument virucides.

## ALCOHOLS

The protein-coagulating action of alcohol requires that instruments be thoroughly cleansed before contact. Providing this is done, disinfection is rapid; sporicidal activity, however, is very slow (Nye and Mallory, 1923). Although the work of Morton (1950) suggests that ethyl alcohol has a high degree of bactericidal activity in the range of 50 to 95 per cent, it would seem desirable to adhere closely to the time-honored concentration of 70 per cent by weight for the disinfection of suitable surgical and dental materials. Isopropyl alcohol is probably more germicidal than ethyl alcohol in comparable concentrations, but most effective at 90 to 95 per cent. With the use of alcohol one must be careful to avoid excessive dilution or evaporation because loss of activity is comparatively rapid when the concentration drops below 50 per cent.

Surprisingly, very little is known about the fungicidal power of the alcohols. There is some indication that spore suspensions are alcohol-resistant, but available data have little bearing upon instrument disinfection.

Alcohol may well prove to be the germicide of choice for acid-fast disinfection (Smith, 1951; Lawrence and Grover, 1951).

Whether the alcohols have any practicable application as virucides is an open question. The highly contradictory nature of pub-



lished reports is no doubt due in part to differences in the concentration of alcohol and in the type of virus studied. The discovery by Olitsky and coworkers (1928) that the addition of small amounts of alkali greatly increases virucidal activity is worth noting with respect to instrument germicides.

### FORMALDEHYDE

A strong aqueous solution (3 to 8 per cent) is actively germicidal and sporicidal in the presence of organic matter (McCulloch, 1945). The disadvantages of formaldehyde are its irritating fumes and the coagulation of protein. In spite of the latter property it can be used to disinfect and sterilize dirty knife blades (Spaulding, 1939). The proper tests for tuberculocidal activity have not been made, but Smith's report (1951) suggests that it may be useful for instruments. Its value as a virucide is uncertain (McCulloch, 1945).

Formaldehyde gas is also actively germicidal and sporicidal in an environment where the relative humidity is 80 per cent or higher. And the inner walls of vessels containing aqueous or alcoholic formalin are sterilized within 5 minutes if only vegetative bacteria are present; it may take as long as 4 hours if there are airborne or pathogenic spores (Spaulding, 1938). It is of interest that Ecker and Pillemer (1938) devised a pressure cooker apparatus for sterilizing instruments with formaldehyde fumes.

### COMBINATION GERMICIDES

At the present time there is an increasing tendency to use combinations of chemicals for instrument disinfection. Most of them contain two or more of the following: formaldehyde, alcohol, hexachlorophene (G-11), a quaternary ammonium compound or a cresol. This is all to the good providing the combination employed is known to be an improvement over its single ingredients. For example, the addition of a surface-active agent may increase germicidal action of formalin-alcohol. The data in table 100 are presented to illustrate this and to show that the best combinations are not likely to be arrived at empirically but must be determined by experimental trial. Although the anionic detergent "Aerosol OT", for example, is not germicidal by itself, it has a significant effect as an additive agent; on the other hand, "Tergitol 7" is a disappointment.

The combination of formalin and an alcohol is definitely superior to either one alone, and the mixture is the most rapidly sporicidal of the germicidal solutions suitable for instrument disinfection. Upon the addition of one of several compounds which may be used,

another increase in activity occurs (table 100). Even dry, heavily contaminated knife blades are sterilized of tetanus spores within a few hours and for relatively clean instruments the sterility time is considerably less.

TABLE 100.—SPORICIDAL ACTIVITY OF COMBINATION GERMICIDES

Test Organism— <i>B. anthracis</i>				Results	
Test Procedure—Wet bloody knife blade					
Germicide				Positive	Negative
% HCHO-68%	ethanol-9%	methanol (by wgt.)		2 hours	3 hours
"	"	" + 1% Aerosol OT		2 hours	3 hours
"	"	" + 2% "		1 hour	2 hours
"	"	" + 3% "		30 minutes	1 hour
"	"	" + 4% "		20 minutes	30 minutes
"	"	" + 5% "			10 minutes
Spore Count—238,000 per blade					
Test Organism— <i>C. tetani</i>					
Test Procedure—Dried bloody knife blade					
% HCHO-65%	isopropanol-3%	methanol (by wgt.)		1½ hours	2 hours
"	"	" + 0.1% G-11		30 minutes	1 hour
"	"	" + 0.5% G-11		15 minutes	30 minutes
"	"	" + 1.0% G-11		15 minutes	30 minutes
"	"	" + 0.75% Tergitol 7		1½ hours	2 hours
% HCHO-65%	isopropanol-3%	methanol + 0.5% G-11		1 hour	1½ hours
Spore Count—207,000 per blade					

## INTERPRETATION OF RESULTS

The value of instrument germicide tests is directly related to the knowledge of the operator and the care with which they are performed. An unrecognized violation of bacteriological technique may not only nullify the results but, what is more important, lead to erroneous conclusions. The most common sources of error are: (1) failure to include adequate bacteriostatic controls; (2) the assumption that spores are always present merely because the culture being used has been inoculated with a sporulating species of bacteria. A striking example of both pitfalls was the inability of Klarmann and Wright (1950) to confirm published results which had been interpreted to mean that cetyltrimethylammonium bromide chloride is sporicidal. It is evident from their data that the original investigator misinterpreted bacteriostasis as a bactericidal effect, and failed to distinguish between "spore-bearing anaerobes" and clostridial spores. Brewer (1939) also called attention to this same type of error.

Not only should each test be designed to approximate a particular condition of usage, but the results are valid only for that situation. A test carried out, for example, with lightly contaminated knife

blades in the absence of blood, serum or pus is intended to duplicate a previously cleansed flat instrument. The results of such tests are not applicable to dried bloody hinged instruments. In 1939 the writer published results of tests made with heavily contaminated knife blades which had been covered with blood or pus and in some instances also dried. The sterility times which appeared in that report have been quoted repeatedly as the number of hours required by the respective solutions to *disinfect clean* instruments. Actually, the values for "clean" blades are much less than the figures being quoted; on the other hand, those for dried bloody hinges are even longer. It should be clearly understood that a single type of test does not provide valid information for all kinds of situations in which instrument germicides are used. The results should only be applied to the type of application which the test is intended to represent.

### THE IDEAL INSTRUMENT GERMICIDE

The perfect instrument germicide is not likely to be found or created as will be seen by examining the following arbitrary criteria for an ideal solution:

1. Produces rapid sterilization of instruments which have been mechanically cleansed but still carry a few tubercle bacilli, spores or virus particles.
2. Retains a major portion of its activity in the presence of body protein and does not coagulate it.
3. The quantity of germicide remaining on instruments after immersion does not irritate tissues.
4. No irritating fumes or unpleasant odor.
5. Does not corrode metal, damage rubber goods or dissolve cement mountings of lens systems.
6. Low cost.

The first criterion listed is the primary one, the others secondary. For example, safety should not be sacrificed; whereas the risk of tissue irritation can be avoided by rinsing in sterile water after disinfection. If the user chooses to ignore the risk of spore and tubercle bacillus contamination, any one of many available solutions will be satisfactory. On the other hand, if emphasis is placed upon protection against tuberculosis, the number to choose from is much smaller. And those who demand a sporicide are limited, for the time being at least, to aqueous and alcoholic formalin solutions.

### DISINFECTION VS. STERILIZATION

The word *disinfection*, by classical definition, means the destruction of disease germs or other harmful microorganisms (but



not spores). Because the great majority of instrument germicides fail to destroy spores at all or require more than 24 hours to do so, *disinfection*—and not *sterilization*—appears to be the proper term to describe the end result of their action. And yet it can be argued that most of them are not even capable of producing *disinfection*. The tubercle bacillus, for example, does not form spores and is certainly a harmful microorganism; thus it logically falls within the category of germs which a disinfectant is supposed to kill. This line of reasoning leads to the conclusion that, as far as the chemical treatment of instruments is concerned, only those germicides which are tuberculocidal can be said to *disinfect*.

Whenever it is used the word *disinfection* should be accompanied by a qualifying phrase to indicate whether or not it is meant to include *tuberculo-disinfection*. Much the same comment can be made about viruses, the difference being that practicable means of measuring chemical virucides simply are not available at the present time. As the term is ordinarily used, there is no implication, one way or the other, as to virucidal activity.

In the surgical sense an instrument is disinfected when all of the *harmful* microorganisms are removed or destroyed. By the same token an instrument subjected to germicidal action can be said to have been disinfected if the residual spores are harmless, but not so if they are capable of producing infection. How one is to determine this under practical circumstances has never been established. Certainly the custom of arbitrarily dividing all microbial species into pathogens and nonpathogens has little meaning in the surgical field. The writer has experienced two instances, for example, in which the usually innocuous sporeforming bacillus, *B. subtilis*, was responsible for fatal septicemia in newborn infants as a consequence of large numbers being introduced directly into the deep tissues. It is conceivable that almost any microorganism can become established in traumatized tissue and do harm whenever the first lines of body defense are bypassed as they are in surgical wounds.

If spores and tubercle bacilli are absent, or have been removed by thorough cleansing of the instrument with soap and water, exposure to most of the standard germicidal solutions for ten minutes produces sterility. Because the latter practice is based upon an assumption, it can not be considered entirely safe. But for that matter neither can the practice of placing instruments in boiling water for 5 or 10 minutes. The writer has encountered instances where spore-bearing knife blades were sterilized more quickly by a strong formalin-alcohol mixture than by boiling water. Tainter and coworkers (1944) report a similar experience. Both the boiling water and chemical procedures are widely used and practical experi-

ence dictates the conclusion that the risk is slight unless there is known danger of tubercle bacilli or spores of *B. anthracis*, *Cl. tetani*, *Cl. welchii* (*perfringens*) or other clostridia of the gas gangrene group.

Nevertheless the only completely safe criterion for chemical disinfection of instruments is absolute sterility. If only the ordinary growing forms of bacteria and fungi are present, this is accomplished quickly. But a very much longer time may be required to destroy tubercle bacilli, chlamydospores of fungi or bacterial spores. However long it may take, the demonstration of complete destruction of microbial life by *adequate* microbiologic methods is valid evidence of sterility whether it be accomplished by heat or by chemicals. Thus the attitude of the United States Department of Agriculture that the term "sterilize" must not be applied to instrument disinfection is without scientific basis. Such a position may be necessary for the proper regulation of proprietary products, but otherwise any attempt to distinguish between the state of sterility produced by heat and that produced by chemicals is sheer semantics. Irrespective of terminology the facts are clear; the only completely safe instrument is a sterile one.

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## 27

# FUNGISTATIC AND FUNGICIDAL COMPOUNDS

### INTRODUCTION

IN THE therapy of human mycotic diseases many drugs and a few physical methods are used. Descriptions of fungistatic and fungicidal agents should name those substances which, by appropriate testing means, have been proven to possess antifungal properties *per se* and not merely curative influences on the human mycoses. The use of Roentgen ray epilation in the treatment of human tinea capitis is an example of this reasoning. The therapeutic endeavor in this case, epilation, is concerned more with the elimination of the diseased part, a kind of radiation surgery, than with the actual killing of the pathogenic organisms.

Similar conditions also hold for the so-called keratolytic agents, the best example of which is salicylic acid. They also perform an amputation of the diseased epidermis, chemical in this case, in the hope of eradicating the infection. Mechanical scraping or sand-papering of nails in onychomycosis are procedures of similar purpose, to partially eliminate the diseased area and also as auxiliary measures to render the infected portion of the skin more amenable to actual chemotherapy. Some agents, such as powders, inhibit the propagation of fungi by preventing skin maceration and hyperhidrosis by altering the ecological conditions. Other substances employed in the treatment of mycoses are mainly palliatives, allaying the symptoms of acute inflammation and irritation. Examples of these are potassium permanganate, aluminum acetate, and, to a certain extent, boric acid. These compounds may possess a slight fungistatic power; however, their chief therapeutic purposes are those of soothing and astringency. Therefore, it should not be assumed that an agent is either fungistatic or fungicidal simply because established usage places it in the medical armamentarium of mycotic therapy.

The mechanism of antifungal action is also not very well understood. There are compounds in use which act merely by stimulating the defense mechanism against the invasive pathogenic processes. The use of resin of podophyllum in the treatment of tinea capitis is an example of this kind of agent (Reiss and Doherty, 1951). The oxidative power of dilute potassium permanganate may serve to neutralize certain skin irritating toxins originating in the metabolism of the fungi. The main avenues of antifungal mechanism seem to be: (1) oxidation, (2) reduction, (3) protein precipitation, (4) enzyme poisoning, (5) deprivation of trace metal action by chelating agents and (6) competitive inhibition.

However, since knowledge of the efficacy of many compounds is gained only through empiricism and clinical observation, a classification of compounds, from the point of view of their possible mechanism of action, is almost impossible. This is true also because many compounds tested *in vitro* and found active fungistatically or fungicidally were unsuitable for actual therapy, since they were primary irritants or potential skin sensitizers. As many of the established antifungal substances, such as sulfur, have been in use since antiquity, the newer ones used in clinical medicine have borrowed heavily from the wide experience and vast research material presented by the plant pathologists. Fatty acids, hydroxyquinoline, benzothiazole, quinones, etc. are but a few examples of this lend-lease program. The antibiotics have not been very useful in the therapy of fungus infection, with the exception of the treatment of actinomycosis.

It may be said that the fact that there is such a multitude and chemical diversity of fungistatic and fungicidal agents constitutes but an expression of their innate antifungal weakness and chemotherapeutic ineffectiveness, rather than a tribute to their potency. In the future a number of fungistatic or fungicidal chemicals may be eliminated to make place for one or a few newer and more specifically acting agents, much the same as the advent of antibiotics has replaced many chemicals used in antibacterial therapy.

The number of antifungal compounds is legion, and there exists a real danger in their uncontrolled use. Underwood *et al.* (1946) tabulated 106 such remedies together with their labeled ingredients. They warned of the very great danger of overtreatment dermatitis as a possible result of the injudicious use of these remedies.

There is an abundance of literature reporting the testing of commonly used antiseptic compounds against the dermatophytes. Many phenolic compounds and surface active agents show only minimal effectiveness in test procedures designed more to evaluate bacteriostasis than fungistasis.

Furthermore, many substances had scanty and insufficient *in vivo* testing or uncontrolled clinical observations as the basis for their report in the literature. The authors were not always able to review the abundance of literature with the necessary critical attitude. The reports, especially those of clinical nature, appear to be governed by current therapeutic use. Compounds become known, successes are reported, then they gradually grow out of style because they are proven to be as unsuccessful as the compounds which are already in use and which they were supposed to replace. In clinical reports, mention of the severity, duration and anamnesis of the infections is very frequently neglected. Many authors use a hit or miss technic, without the use of proper treatment controls, without regard to the frequent natural remission of the infection, and with disregard of the value of auxiliary procedures in their reports of therapeutic success.

The picture is one of confusion, necessitating a pessimistic view of the present state of antifungal therapy. The problem in this therapeutic endeavor in its condensed form is that the toxicant must come in contact with and penetrate the outer layer of the fungus in all its phases of growth, sporulation or mycelial growth, without being harmful to the host. Until an antimycotic substance is found which will overcome this barrier our efforts in the therapy of the mycotic diseases will be rather complicated.

The following is a listing, with a few pertinent references, of known antifungal agents. Those used in the treatment of generalized mycoses are mentioned first. Agents acting fungistatically or fungicidally on the superficial mycoses are listed subsequently, according to their chemical relationship.

## ANTIFUNGAL DRUGS USED IN SYSTEMIC MYCOSES

### IODINE

Iodides constitute one of the oldest remedies in the treatment of systemic fungus infections. They may be especially useful in the therapy of generalized moniliasis, North American blastomycosis and sporotrichosis. The iodide may be given as a saturated solution of potassium iodide, or tincture of iodine in milk or water by mouth, sodium iodide intravenously, or as ethyl iodide by inhalation. The latter method delivers the iodine through metabolic transformation to the target organs, especially to the lung tissue in pulmonary mycoses.

### SULFA DRUGS

Sulfadiazine has been found especially useful against species of *actinomyces*, which are closer in their chemical and structural



characteristics to bacteria than to the yeast-like fungi and the dermatophytes. Good results have also been reported on sporotrichosis. North American blastomycosis does not respond. There are reported cases of apparent cure of histoplasmosis with sulfathiazole (Curtis and Cawley, 1947).

Keeney *et al.* (1944) found that sulfathiazole, sodium sulfadiazine and sodium sulfamerazine, in concentrations of 500 to 1000 mg per cent, exhibited both fungistatic and fungicidal effects upon *Actinomyces bovis* in thioglycollate media.

Reilly and Altman (1948) reported a case of cryptococcosis which responded to sulfadiazine therapy. The life expectancy of rats infected with the organisms isolated from this patient increased from 11.4 to 22.6 days under treatment with sulfadiazine.

Dimond and Thompson (1942) tested various sulfonamides *in vitro* on Czapek's agar against *Trichophyton* and found them to be ineffective. Promin, a sulphone derivative, has shown therapeutic effects in histoplasmosis, and stilbamidine, an aromatic diamidine, gave beneficial response in cases of systemic blastomycosis but not in histoplasmosis (Elson, 1945; Schoenbach, 1952). There is an interesting report of treatment of disseminated histoplasmosis in infants with ethyl vanillate (Christie *et al.*, 1951).

## PENICILLIN

Sanders (1946) found penicillin to exert no influence on pathogenic fungi. Only actinomycosis infections respond to penicillin therapy. No therapeutic results were obtained in sporotrichosis, coccidioidomycosis, moniliasis, blastomycosis (North American), histoplasmosis or cryptococcosis.

Neither sulfonamides nor penicillin were therapeutically effective in a case of maduramycosis (Dostrovsky and Sagher, 1948).

## STREPTOMYCIN

Reilly, Schatz, and Waksman (1945) compared the effects of several antibiotics against *Candida albicans*, *Trichophyton mentagrophytes* and *Cryptococcus neoformans*. Streptomycin was less toxic for these organisms than clavacin, gliotoxin, actinomycin, fumigacin, streptothricin or chetomin. Gliotoxin was the most potent of the tested substances. Some case reports on the clinical effectiveness of streptomycin in the treatment of actinomycosis can be found in the literature.

## AUREOMYCIN, CHLORAMPHENICOL, OXYTETRACYCLINE

These broad spectrum antibiotics have been used successfully in the treatment of actinomycosis and nocardiosis (Strauss, Klig-

man, and Pillsbury, 1951). Other antibiotics have been isolated and tested for potential antifungal potency.

#### ADDITIONAL ANTIBIOTICS

Crude tomatine, an extract from *Lycopersicon pimpinellifolium* leaves, does not inhibit the growth of *Candida albicans*, whereas crystalline tomatine is a moderately potent inhibitor. Rutin and quercetin, ingredients of the crude tomatine extract, neutralize the antifungal effects of the crystalline tomatine (Ma and Fontaine, 1948).

Prodigiosin, a water insoluble red pigment produced by *Serratia marcescens*, was found to be fungistatic for *Coccidioides immitis* in dilutions through 1:500,000 (Lack, 1949). It was tried clinically by Wier *et al.* (1952). The results were encouraging but equivocal.

Bacillomycin, an antibiotic from *Bacillus subtilis* was found active against pathogenic fungi but not against bacteria by Landy *et al.* (1948). Subtilin, another antibiotic principle recovered from *B. subtilis* has some fungistatic effect against dermatophytes but less against *Candida albicans* (Moore and Wooldridge, 1950). Other extracts from the same organism with antifungal effects are Eumycin (Johnson and Burdon, 1946), and extract XG, XY (Lewis, Hopper, and Shultz, 1946). *Streptomyces fradiae*, the organism that produces neomycin, also forms an antibiotic which is active in broth upon fungi. It has been designated as fradicidin (Swart, Romano, and Waksman, 1950).

The action of clavacin on dermatophytes was investigated by Loewenthal and Tolmach (1947). There are reports on fungicidin (Hazen and Brown, 1951) and protoanemonin (Conan and Hyman, 1950). However, the antimycotic performance of most of these agents is not very impressive. Future development might eventually furnish a powerful antifungal inhibitor from this group of compounds.

Against some of the systemic mycoses certain antibiotics and chemotherapeutic agents have shown some effectiveness and vice versa, some of the broad spectrum antibiotics have been accused of causing a wider dissemination of an already present fungus infection, mainly *Candida albicans*, even producing fatalities in some cases. Disturbance of the biological balance between bacterial flora on the one hand and the fungus, growing as a non-pathogenic parasite, on the other hand, favors the multiplication and eventual pathogenicity of previously harmless microorganisms by the suppression of the growth of one of the component parts of the biostatic

milieu. To curtail this possibility one of the antibiotics, aureomycin, contains fungistatic substances in the form of methyl- and propyl-para-hydroxy-benzoic acid derivatives (methylparaben, propylparaben) (McVay and Sprunt, 1951). The argument brought forward by Kligman (1952) that the *Candida* infections, though facilitated by the broad spectrum antibiotics, are only of secondary importance, because, among others, they do not mimic completely the pathological picture of the classic infection, is not considered valid. Similarly, the altering of the classic course of events in lobar pneumonia by antibiotic therapy does not cast doubt as to the correct diagnosis and the presence of the disease process.

## ANTIFUNGAL DRUGS IN SUPERFICIAL MYCOSES

### SULFUR-CONTAINING COMPOUNDS

Sulfur in its sublimated inorganic form is often a component part of antifungal ointments. Since, in this form, it is quite insoluble and may act more as a keratolytic agent than as an antifungal substance, the sulfur atom has been incorporated in many organic radicals to make it more readily biologically available to exert its antifungal influence. The inclusion of any compound in this category, however, is not meant to be a tacit admission that the mechanism of its antifungal action is due to the sulfur atom.

Thiourea and its derivatives, particularly hydrazine-dithiocarbamyl, were tested *in vitro* against pathogenic fungi by Danowski and Tager (1948). The compounds were ineffective in experimental infections of mice, though very active *in vitro*. This activity was inhibited by serum (Tager and Danowski, 1948).

Thiocarbamates have been used extensively as fungicides in the control of plant infections. They were first described in the patent literature in the early 1930's. Hopkins *et al.* (1946) investigated tetraethyl-thiuramdisulfide, which at first gave good clinical response, but which later did not compare favorably with other agents. Since no data on the concentrations employed are presented in this report, the mixed results might be an expression of the unusual toxicity curve with two maxima reported by Dimond *et al.* (1941) with tetramethyl-thiuram-disulfide.

Kligman and Rosensweig (1948) tested these compounds in the form of the calcium, the ferric and the sodium dimethyl thiocarbamate against *Trichophyton* and *Microsporon in vitro* and found them quite effective and not inactivated in the presence of blood. They also reported a low skin sensitization. Kligman and Anderson (1951) used zinc ethylene bisdithiocarbamate for evaluation of the local treatment of tinea capitis. Mercaptobenzothiazole



was found useful in plant diseases due to fungi. A new derivative of benzothiazole, 2-dimethyl, amino-6-(p-diethylaminoethoxy)-benzothiazole dihydrochloride, was selected for intensive laboratory study on dermatophytes by Grunberg *et al.* (1950). This compound possesses good fungistatic and moderate fungicidal activity against the dermatophytes. In its use in tinea capitis, cases of alleged neurotoxicity, giving rise to convulsions in children, have been reported (Hitch, 1952). Kligman and Anderson (1951) in a careful evaluation of local treatment methods of tinea capitis, came to the conclusion that an unmedicated carbowax mixture furnished about the same percentage of cures (63 per cent) in this disease as had been reported with the use of some medicated ointments. Therefore, the correct evaluation of a new fungicide designed for the treatment of tinea capitis by clinical statistics is often very difficult.

Phenothiazine, which the plant pathologists have found to be fungicidal due to its oxidized form phenothiazone (Goldsworthy, Carter, and Green, 1939), was clinically tested by Hopkins *et al.* (1946) with a low percentage of satisfactory results.

The reported variable antifungal action of certain antihistamines depended on their molecular structure. Those most effective contained a phenothiazine nucleus (Mitchell, Arnold, and Chinn, 1952).

Derivatives of thioglycollic acid have a softening influence on keratin (Goetz, 1950). They are not contributory, in the authors' experience, to facilitating a more satisfactory fungistatic effect of antifungal substances on either hair or nails.

The sulfa drugs, as mentioned before, have no therapeutic effect on the superficial mycoses. Sodium thiosulfate in 50 per cent solution in 5 or 10 per cent glycerine has been reported clinically effective (Hoffman, 1948). However, *in vivo* tests have not shown any considerable antifungal activity.

#### HALOGEN-CONTAINING COMPOUNDS

Tincture of iodine is one of the oldest household remedies in use against superficial mycoses. Iodine is also incorporated in an ointment base and used in the treatment of tinea capitis. Unfortunately, in the concentrations in which it is being used it is quite irritating to the skin. Furthermore, as with most halogen containing compounds, the antifungal effect of iodine is, to a great extent, inhibited in the presence of serum or blood. To circumvent this effect, a multitude of organic compounds have been prepared with the purpose of carrying the effective halogen through the protein barrier of the skin into the fungus cell. Some of these substances

ave proved quite irritating; others exert their main therapeutic activity in the realm of antiseptics. Chlorthymol has high fungistatic power which is severely diminished in the presence of serum. Ethyl chloride spray was tested clinically on tinea type lesions (Bograd, 1943). Trichlorphenol was clinically effective but caused frequent skin irritation (Hopkins *et al.*, 1946).

Monopyridine iodine, (I) p-nitrobenzoate, was investigated by Kleinberg and Meyer (1946) *in vitro* for activity against *T. gypsum* and *M. audouini*. A fungistatic effect could be shown in the absence of serum. A combination of the antifungal action of sulfur, the halogens, and the utilization of the mechanism of chelation by combining with copper may be effective in the therapeutic potentialities of 2,2'-dihydroxy-5, 5'-dichlorodiphenylsulfide or D25 (Schraufstaetter, Richter, and Ditscheid, 1949). Richter (1950) reported good therapeutic results in superficial mycoses. No results were obtained in tinea capitis. The compound may also be administered effectively by mouth. The *in vitro* tests were performed in a liquid medium. Addition of serum resulted in a marked reduction of activity, a result which could be confirmed by the authors' own experiments. A mixture of cetyl trimethylammonium, a quaternary ammonium compound, and pentachlorophenate has been used in the treatment of tinea capitis. Sullivan and Bereston, (1952), in a comparative study of thirteen compounds commonly used in the therapy of this disease found 84 per cent treatment failures with an ointment containing pentachlorophenate as an active ingredient. They found 5-chlor-salicylanilide the most effective agent producing 3.1 per cent cures in a group of 108 patients.

#### METAL-CONTAINING COMPOUNDS

Boric acid in powder form is an old standby in the treatment of superficial mycoses. It was frequently prescribed in glycerine for the treatment of thrush in infants. Copper, a known plant fungicide, has been administered to the human skin by means of iontophoresis of copper sulfate solutions. However, its administration was found to be ineffective (Gabrielsen and Sylvest, 1945).

The copper ion has been incorporated into some organic radicals and tried mainly in the therapy of tinea capitis. These are copper oleate, (Carrick, 1946), copper naphthenate, and copper undecylenate. Copper-8-quinolinolate may be used as a preservative for fabrics and leather against *Trichophyton interdigitale* (Benignus, 1948). One may assume that the copper is the active principle of this combination and not quinoline. Kleine-Natrop (1948) found inorganic copper salts to have an oligodynamic effect

on fungi which might explain the therapeutic results in experimental infections of guinea pigs with *T. gypsum*.

Mercury salts, both inorganic and organic derivatives, have a fungistatic effect. Sublimate or mercuric chloride has fallen into disuse because of its potential irritating qualities and toxicity in the prolonged therapy of superficial mycoses. Ammoniated mercury ointment from aminomercuric chloride is an old remedy in skin diseases and has also been applied with reported success in dermatophytosis and tinea capitis (Miller, Lowenfish, and Beattie, 1946). Organic mercury compounds, such as merthiolate and metaphen, are used occasionally, but it is demonstrable that their effectiveness could be curtailed *in vitro* by the addition of sulfhydryl compounds to the media (Brewer, 1940). Their main therapeutic aim is, however, the disinfection of the skin surface, not the treatment of superficial mycoses. Robinson (1948) grew cultures of *M. audouini* from hair treated with merthiolate 1:1000. Phenyl mercuric chloride, -nitrate and -acetate have, nevertheless, been found effective in the treatment of various cases of human dermatophytosis (Byrne and Croxon, 1944).

Nickerson and Chadwick (1946), in studying the metabolism of dermatophytes, found 1/100 molar zinc chloride to completely suppress their respiration. No valid attempts were described to study the influence of proteins or to inactivate the zinc adherent to the fungus, thereby ascertaining the eventual reversibility of the process. A bimodal curve of activity resulted from the addition of increasing amounts of sodium chloride, similar to the suppression of toxicity of mercuric chloride and copper sulfate by potassium chloride, investigated by Clark (1901). However, with the exception of tinea cruris and tinea glabrosa, treatment of actual clinical infections was unsuccessful with a 1 per cent solution of zinc chloride (Dolce and Nickerson, 1947). Soo-Hoo and Grunberg (1950) investigated the antifungal activity of metal derivatives of 3-pyridinethiol *in vitro* without serum. Reiss and Doherty (1950) describe the clinical use of pyridyl-3 zinc mercaptide, the most effective of the group, and resin podophyllum in fungus infections.

Silver nitrate solution is recommended for the treatment of dermatophytosis. The rationale derives only from clinical observation. A silver complex salt, ammoniacal silver nitrate, was recommended by Nickerson and White (1948) in the treatment of fungus infections of the nails. In the hands of Franks and Sternberg (1950), this therapy was without benefit in 18 patients with onychomycosis.



## ALIPHATIC COMPOUNDS

Formaldehyde is used chiefly as a disinfectant to prevent reinfection from shoes in cases of dermatophytosis under treatment. Clark (1899 *a, b*) tested the inhibitory effect of fatty acids on fungi. Kiesel (1913) found that the fungistatic activity of the fatty acids increased with the number of carbon atoms up to 11 or 12. Undecylenic acid was the most active compound. Sodium and calcium propionate have long been incorporated in bread dough and cake batter by many large bakeries as nontoxic inhibitors of potential mold growth. Peck and Rosenfeld (1938) studied the antifungal effect of the fatty acids and their salts on dermatophytes and reported encouraging clinical results with sodium propionate, a constituent of sweat. Keeney *et al.* (1945) recommended the sodium salt of caprylic acid as more effective than sodium undecylenate. Critical *in vitro* studies by Oster and Golden (1947) proved both propionic acid and undecylenic acid to be only weakly fungistatic. Clinical experience in recent years has also confirmed these shortcomings. Mitchell (1948) was able to grow cultures of *M. audouini* from infected hairs in an ointment containing as active ingredients propionic and caprylic acids and in another ointment containing undecylenic acid. Rothman *et al.* (1945) observed that the hair of children contained only a fraction of the small amounts of fatty acids present in the hair of adults. These findings provoked Rothman to postulate the theory that the prevalence of tinea capitis in children is due to the lack of antifungal fatty acids in their hair fat. However, the amounts contained in the hair sebum of the adult are not at all fungistatic, and it is difficult to comprehend how they could prevent an infection. Kligman and Ginsberg (1950) have also attacked the basis for this unsubstantiated hypothesis which already has found wide acceptance in the pertinent literature. Bereston (1947) suggests propyleneglycol dipropionate as a fungicide in a 100 per cent concentration, the compound allegedly interfering with the fat phase of the fungus metabolism due to the "principle of unfamiliarity." A similar compound, supposedly displaying competitive inhibition, propylene glycol dipelargonate, gave 58 per cent of treatment failures in cases of tinea capitis (Sullivan and Bereston, 1952). In the authors' opinion, it would seem to be a testimony of antifungal weakness, to say the least, when the topical application of any compound must be in the percentage range of 20 per cent and above to be clinically effective.

## AROMATIC COMPOUNDS — PHENOLIC COMPOUNDS AND DERIVATIVES

Salicylic acid together with benzoic acid is one of the oldest and most commonly used remedies against dermatophytosis. In a

percentage of 6 per cent salicylic acid and 12 per cent benzoic acid in an ointment base it is known as Whitfield's Ointment. Its antifungal power *per se* is practically non-existent; its action is due chiefly to its keratolytic effect, and it is highly irritating. The ointment is available in half strength and third strength. It constitutes a typical example of an extremely impotent antifungal compound used in high concentrations (sometimes double strength) (Montgomery and Casper, 1945) for the therapeutic action it probably cannot accomplish, unless one takes salicylic acid out of the class of antifungal compounds and ascribes its action to keratolysis. Even then, it is difficult to understand why such enormous concentrations of the compound are being used. Benzoic acid, on the other hand, has good fungistatic qualities according to Hopkins *et al.* (1946). Phenol and resorcinol have been tested *in vitro* and were not found to be fungicidal in concentrations as high as 15 percent (Golden and Oster, 1947). The use of phenol and camphor mixtures, which liquefy when rubbed together, was at one time recommended for the treatment of dermatophytosis (Glenn and Hailey, 1943). Many skin irritations were reported following unsupervised self-medication with this mixture.

Other derivatives of phenol have been suggested for the treatment of dermatophytosis. Most of them, however, have been abandoned or were very short-lived, because of either insufficient activity or excessive skin irritation. They are enumerated here merely for the sake of completeness. Weirich and Pokorny (1942) recommended cresol as an antimycotic remedy. A derivative of cresol, metacresylacetate, must be used in 80 to 100 per cent concentration to be clinically effective. Tetrabromcresol was clinically tested by Hopkins *et al.* (1946). Halogen derivatives of phenol were tested for fungicidal activity by Klarman *et al.* (1934) and recently by Walker, Porter and DeKay (1952). Hartley (1947) recommended p-chlorophenyl glycerolether. Thymol was studied by Stovall *et al.* (1935). The fungicidal properties of quinones were the subject of a patent issued as early as 1930 to Brodersen and Ext. Again borrowing a leaf from the plant pathologist, tetrachloro-p-benzoquinone (Cunningham and Sharvelle, 1940) was tested for the treatment of dermatophytoses (Hopkins *et al.*, 1946) and tinea capitis (McGavack *et al.*, 1949). The results were encouraging. In the authors' experience, however, derivatives of benzoquinone and naphthoquinone are highly irritating to the skin, a quality which precludes their clinical application, despite their outstanding *in vitro* activity.

Another phenolic compound is salicylanilide, which was used extensively by the U. S. Public Health Service in the treatment of

inea capitis in an outbreak in Hagerstown in 1944-45. (Schwartz *et al.*, 1946). However, Robinson (1948) could not find any fungicidal effect in *in vitro* studies on affected hair. Sullivan and Bereston (1952) found 74 per cent failures with this compound.

Halogens were introduced into the ring of salicylaldehyde by Felton and Brewer (1947) to increase fungistasis. However, Sullivan and Bereston (1952) report 80 per cent treatment failures in tinea capitis with one of these compounds, dibrom-salicylaldehyde. Methyl and propyl-hydroxybenzoate show *in vitro* effects inhibitory to the growth of *C. albicans*. They were also found to be of value in preventing the rampant, unhampered growth of *C. albicans* associated with aureomycin therapy (McVay and Sprunt, 1951). Hillegas and Camp (1945) tested 2-chlor-4-phenyl phenol *in vitro* and found it more effective than undecylenic acid. 4,4'-isopropylidene diphenol was tested by Hopkins *et al.* (1946). Hexylresorcinol has also been tried *in vivo*. The compound has fungistatic properties which are curtailed by the addition of a minute quantity of lecithin (Leão and Eichbaum, 1948). Nitrophenols have been reported to exhibit clinical effectiveness against dermatophytosis; 2-chlor-, 2-brom-, and 2-iodo-, 4 nitrophenol were tested. Whether the nitro group or the halogen is the active group in these compounds should be further investigated. Woodward, Kingery and Williams (1933, 1934) studied the fungicidal power of various halogen and alkyl substitutions on the phenol ring *in vitro*. It was found that, increasing from chlorine through bromine to iodine, the halogen atoms increased the antifungal potency from 4 to 10 times, whereas nitro groups introduced into the phenol ring had no augmentative effect. The nitro group introduced into the furan ring as 5-nitro, 2-furfuryl methyl ether and 5-nitro 2-furaldehyde oxime exhibited good fungistatic activity *in vitro* in the absence of serum (Ward *et al.*, 1948). 2,4-dinitro orthocyclohexylphenol was recommended for the treatment of dermatophytosis by Hopkins *et al.* (1946), but it caused skin irritation and staining.

#### NAPHTHOL AND QUINOLINE COMPOUNDS

Gonzalez (1945) reported on the inhibitory effect of quinones on the growth of *Penicillium notatum*. Colwell and McCall (1946) investigated the mechanism of fungus growth inhibition by 2 methyl 1,4 naphthoquinone. They concluded that the growth inhibition of this compound occurs by its combination with sulfhydryl groups essential for growth. A similar mechanism was postulated for the activity of many antibiotic substances. Golden and Oster (1946) found the highest fungistatic activity, according to the Burlingame-



Reddish test method, exerted by 2 amino 1,4 naphthoquinonimine HCl. Subsequently, Kligman and Rosensweig (1948) obtained similar results with other naphthoquinones. One of the best remedies for the treatment of fungus infection, according to Jadassohn and collaborators (1944, 1947), is a derivative of 8-hydroxyquinoline. The most effective compounds found were 5,7 dichlor, 8-hydroxyquinoline and 4,7 dichlor, 8-hydroxyquinoline. Sigg (1947) reported favorable clinical results with the first compound. Oster and Golden (1948) in examining twenty-four derivatives of quinoline, found only 8-hydroxyquinoline benzoate active in fungistatic and fungicidal tests. The possible mechanism of action of 8-hydroxyquinoline, according to Zentmyer (1944), is due to its possible chemical inactivation by chelation of trace metals necessary for the growth of the fungus, either in the nutrient medium or in the cell of the fungus. An antifungal preparation containing 2.5 per cent of 8-hydroxyquinoline benzoate was used successfully in the treatment of dermatophytosis (Oster and Golden, 1949).

## DYES

These substances have long standing use in the dermatological armamentarium. Best known in fungus therapy are gentian violet, effective especially against *C. albicans*, and Castellani's paint, a compound solution of carbolfuchsin and phenol. Riley and Flower (1950) subjected both dyes to a critical comparative evaluation of their *in vitro* antimycotic activity. They found that gentian violet solution will inhibit the growth of *C. albicans* in a dilution of 1:1,000,000, while inhibition with Castellani's compound solution of carbolfuchsin begins at a dilution of 1:100. They conclude that factors other than antifungal activity may account for the allegedly good clinical activity of Castellani's compound solution of carbolfuchsin.

Hillegas and Camp (1945) found by *in vitro* testing a fungistatic action of brilliant green and gentian violet superior to that of undecylenic acid. The dyes have the disadvantages of poor cosmetic qualities and of potential spoiling of clothes and laundry when applied to the skin of patients.

## QUATERNARY AMMONIUM COMPOUNDS

Reiss and Lustig (1948) evaluated the fungicidal activities of various quaternary ammonium compounds, phenols, undecylenic acid and their mixtures *in vitro* with a dilution method, because of the non-diffusibility of the detergent compounds into agar. They

found no synergistic action of the quaternary ammonium compounds on other antifungal substances. Similar observations were also made by Miller, Lowenfish, and Beattie (1946) testing the effect of dioctyl sodium sulfosuccinate and sodium tetradecylsulfate. Hopkins *et al.* (1946) found alkyl-dimethyl-benzylammonium chloride (Zephiran) and hexadecylpyridinium chloride (Ceepryn) to be fungistatic rather than fungicidal *in vivo* and to be rather ineffective clinically. Walker, Porter and DeKay (1952) report on the particular antifungal activity of laurylpyridinium chloride.

## SEX HORMONES

It has been assumed that sex hormones might have fungistatic effects. Lewis, Hopper and Reiss (1946) demonstrated that neither estrogens nor androgens, though effective *in vitro* (Reiss, 1947) were successful clinically in tinea capitis. Reiss (1949) investigated the effect of twelve steroid compounds in relation to their fungistatic and genestatic (sporulation-preventing quality) potencies. He found no correlation between their physiological and fungistatic effects. The greatest fungistatic potency was exerted by diethyl stilbestrol, a diphenol compound which had already been investigated together with other diphenols such as salicyl and benzenestrol by Heinemann (1947).

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## 28

# FOOD PRESERVATIVES

IN A broad sense, a food preservative may be regarded as any agent, chemical or physical, which extends the storage life of foods or food products by retarding or preventing changes in flavor, odor, color, nutritive value, texture or consistency, or appearance. The present discussion, however, will be limited to chemical agents and specifically to those which inhibit or destroy spoilage types of microorganisms, and to those which retard or help to prevent undesirable changes caused by enzymes or other factors.

### NATURE OF SPOILAGE

Spoilage of foods and food products is largely the result of microbial, enzymatic and chemical actions. The enzymes possessed by the microorganisms and those present in foods are particularly important in such deteriorative changes.

Spoilage may be evidenced by changes in the odor, flavor, consistency, color, appearance or nutritive value. Odor changes may be caused by the production of volatile or aromatic compounds, such as acids, aldehydes, amines, sulfides, mercaptans, and ketones. Flavor changes may be due to the formation of any of the foregoing substances, to the presence of microorganisms or to the utilization or alteration of normal ingredients through fermentation, proteolysis, or lipolytic action (oxidative or ketonic rancidity). Changes in consistency may result from solubilization of proteins, hydrolysis of starches, curd formation in milk, rope formation in bread, softening of pickles or sauerkraut, hardening of citrus fruits, slime formation, leek of strawberries, the rotting of apples and citrus fruits, and other factors. Color changes may be attributed to the growth of pigmented or pigment-producing organisms, or to the deterioration of the natural color due to oxidative or other types

of action. Alterations in appearance may be brought about by changes in consistency and color. Changes in food value may be induced by destruction or loss of vitamins or the utilization of food ingredients by microorganisms with the production of materials of lower or no value.

For extended discussions on the microbiology of spoilage, the reader is referred to the publications of Tanner (1944), Jensen (1945), Hammer (1948), Elliker (1949), Prescott and Dunn (1949), Sarles *et al.* (1951), and others.

## CHEMICAL AGENTS USED TO PREVENT THE GROWTH OF SPOILAGE MICROORGANISMS

### GENERAL CONSIDERATIONS

The use of chemical agents to inhibit or prevent deteriorative changes in foods is subject to rigid control by government authorities. State and federal agencies, including the Food and Drug Administration, Federal Security Agency, regulate the addition of chemicals to foods and should be consulted by a food manufacturer before using any compound with which he is not familiar. Regulations are promulgated by government agencies in the interests of both the manufacturer and the consumer.

There are a number of requirements for the chemical food preservative. Most important of all is the question of toxicity. The preservative must be non-toxic, or relatively so, in the concentration employed. It should effectively prevent the growth of spoilage and desirably other types of microorganisms by virtue of inhibitory and germicidal action. It should be free of color, odor, and flavor in the dilutions used. It should be soluble in water (sometimes in oil), noncorrosive and low-priced. It should be stable during storage and capable of sustained action when in use. Obviously, these requirements limit greatly the number of chemical agents which may be employed as food preservatives.

Acids, salts, and sugars are the principal food preservatives of a chemical nature employed in this country. Acids, chiefly lactic, but occasionally including propionic and/or acetic, are produced during controlled fermentation in special types of food materials, including sauerkraut, pickles, olives, fermented milks, and cheeses. Benzoic acid, sulfur dioxide, and certain salts, including sodium benzoate, sodium and calcium propionates, sodium diacetate and sodium chloride, may be added to foods to prevent spoilage. Sugars are employed in the manufacture of jellies, jams, preserves, sweetened condensed milk, sweet pickles, and other products and aid in the preservation of the products in which incorporated. Other

chemical compounds are used in special situations to retard the development of spoilage microorganisms.

It is not permissible to add antibiotics to foods or food products at the present time, but their use is being studied and evaluated.

## ACIDS

A considerable amount of research has been carried out on the germicidal and antiseptic properties of acids. Among the significant reports and reviews of this subject may be listed those of Reid (1932), Cowles (1941), Levine and Fellers (1940), Nunheimer and Fabian (1940), Erickson and Fabian (1942), Shillinglaw and Levine (1943), Rahn and Conn (1944), McCulloch (1945), Porter (1946), and Von Schelhorn (1951).

Acids owe their germicidal and inhibitory activities to both the dissociated fractions and to the undissociated fraction of the molecule. Mineral acids are highly dissociated and consequently produce a low pH level which is unsuited to the growth or survival of many species of microorganisms. Organic acids are less dissociated and an important part of their action may be attributed to the toxic effect of the whole or undissociated molecule. The anions of the various acids undoubtedly contribute in some measure to the overall effects.

Data of general interest relating to organic acids are presented in the accompanying table from the report by Reid (1932).

TABLE 101.—BACTERICIDAL ACTION OF ORGANIC ACIDS AGAINST VARIOUS ORGANISMS IN 15 MINUTES AT 20° C.\*

### Bactericidal dilutions

Acids	<i>Ps. aeruginosa</i> ( <i>B. pyocyaneus</i> )	<i>S. typhosa</i> ( <i>B. typhosus</i> )	<i>E. coli</i> ( <i>B. coli</i> )	<i>M. pyogenes</i> var. <i>aureus</i> ( <i>Staph. aureus</i> )
Monobasic				
Acetic	N 3	N 2	N 1.5	1.5 N
Propionic	N 4	N 4	N 2	N
Butyric	N 8	N 8	N 5	N 3
Valeric	N 20	N 20	N 14	N 14
Hydroxy monobasic				
Glycollic	N 35	N 16	N 6	N 2
Lactic	N 30	N 14	N 4	N 1.2
Dibasic				
Oxalic	N 320	N 84	N 36	N 18
Malonic	N 120	N 30	N 26	N 5
Succinic	N 5	N 3	N 2	N +
Tribasic				
Aconitic	N 60	N 35	N 10	N 10
Citric	N 18	1.5 N	2.5 N	3 N

\* Reid, J. D., 1932, Am. J. Hy. **16**, 540-556.

+ indicates growth



## LACTIC ACID

Lactic acid ( $\text{CH}_3\text{CHOHCOOH}$ ) occurs naturally in a number of fermented food products. It is the principal acid found in sauerkraut and in fermented pickles, olives and milks, and is a most important acid in cheese making. The lactic acid present in some of these products aids materially in their preservation.

Lactic acid is formed from suitable carbohydrate substrates, such as the lactose in milk, the sugars in molasses and hydrolyzed starches, by species of the lactobacilli, streptococci, leuconostoc, coliform bacteria, molds (*Rhizopus* and *Mucor*, in particular), and other microorganisms. The spoilage of certain types of foods, as the result of the souring brought about by microorganisms, is of common occurrence unless the foods are properly protected.

Lactic acid production in fermentation media inhibits the development of putrefactive types of bacteria and butyric acid anaerobes in particular. It will also inhibit the growth of other types of microorganisms as the result of the production of an unfavorable pH in the substrate.

Lactic acid formation may, on the other hand, encourage the development of those types of microorganisms which grow best in the pH range produced by the lactic acid bacteria. Many species of yeasts and molds will grow particularly well in foods containing lactic acid, unless special precautions are taken to prevent such growth. Precautions may include control of sanitation, temperature, salt concentration and distribution, and the use of special methods, as for example, the employment of ultraviolet light, mustard oil, mineral oil, or other adjuncts to prevent the growth of film yeasts and molds on the surfaces of products such as sauerkraut and pickles.

Lactic acid, produced by fermentation, may be added as an acidulant to food materials to produce the desired pH or acidity. It is used in certain carbonated beverages to lower the pH to a level where the development of spoilage microorganisms will be inhibited (aided by the use of carbon dioxide under pressure). It is used in the making of pickles and in adjusting the reaction of fermentation media in the manufacture of ethyl alcohol, compressed yeast and other products. In each of these cases the lactic acid aids in the retardation of the growth of undesirable microorganisms.

*Sauerkraut*.—Sauerkraut is the food product made from shredded cabbage by the use of salt and controlled fermentation. The acids and anaerobic condition produced by the action of lactic acid bacteria on the juices withdrawn by the salt preserve the sauerkraut.

Salt in a concentration of 2 to 2½ per cent, average about 2¼ per cent, is added to shredded cabbage and thoroughly mixed with it. The salt accelerates the removal of moisture from the cabbage which contains dissolved sugars, nitrogen-containing substances, minerals, vitamins, and other compounds. These ingredients act as the growth medium for special types of microorganisms resident on the shredded cabbage.

Lactic acid bacteria are largely responsible for the characteristic flavor and appearance of the sauerkraut. Pederson (1930) and others have shown that in a normal fermentation species of lactic acid bacteria develop in sequence. Gas-producing cocci, exemplified by *Leuconostoc mesenteroides*, develop first, giving rise to 1 to 1.3 per cent of lactic and acetic acids, carbon dioxide, ethyl alcohol, and mannitol. These bacteria are eventually superseded by non-gas-producing rods of the *Lactobacillus plantarum* type which produce lactic acid almost exclusively from sugars and mannitol, and gas-producing rods of the *L. brevis* type which form additional quantities of lactic and acetic acids, ethyl alcohol, carbon dioxide and mannitol. The final acid concentration should be a minimum of 1.5 per cent and may amount to 2 per cent or more.

The acids produced inhibit the growth and activity of putrefying types of bacteria. However, should the lactic acid be consumed by the development of film-forming yeasts or molds, then spoilage by microorganisms normally inhibited by 1.5 per cent or so of acid may take place.

Many factors, such as the variety of cabbage; the kind, amount and distribution of the salt (Pederson, 1946); the cleanliness of the fermentation vat; the temperatures maintained during fermentation; and the control of growth of undesirable microorganisms in the brine, have important effects on the quality of the final product (Prescott and Dunn, 1949). Salt in the correct concentration and uniformly distributed aids in selective development of lactic acid bacteria. The anaerobic conditions produced in the vat and the control of fermentation temperature favor the growth of these organisms also. Prevention of the growth of film-forming yeasts and molds on the surface of the brine through the use of radiant energy, mustard oil, mineral oil, and careful sanitation is important in the production of a quality product.

*Cucumber Pickles.*—Cucumbers are an item which may be preserved in a number of different ways: (1) through the action of sodium chloride in a 60° to 70° brine; (2) through the combined action of salt and the acids produced in the brine as the result of bacterial fermentation; (3) through the addition of vinegar to washed salt stock, as in the manufacture of sour pickles; (4)

through the addition of vinegar, sugar, and spices to salt stock, as in the making of sweet pickles; and (5) by other methods, including heat processing.

Salt stock, which may be used for the preparation of sour pickles or sweet pickles, is prepared as follows. Selected cucumbers, graded for proper size, are placed in vats, which have been carefully cleaned and sanitized, and covered with a brine having an initial salt concentration of 2.5 to 10 per cent. They are kept submerged by placing a weighted cover over them. During a period of several weeks, the concentration of sodium chloride is progressively increased to a final one of usually 15 to 16 per cent or higher. At the latter salinity, the salt stock may be stored for months without serious danger of spoilage.

During the early part of the brine treatment, sugars and other soluble substances are withdrawn from the cucumbers by osmosis. These sugars are acted upon by lactic acid bacteria, occasionally gas-forming *Aerobacter* species, and yeasts. The acids produced by the lactic acid bacteria, principally lactic acid, are desirable and inhibit the development of putrefactive and other types of bacteria. Lactic acid formation occurs most rapidly at optimum temp. when the salt concentration is lowest. Acid is formed more slowly and in smaller amounts when the initial salt strength is higher. The presence of *Aerobacter* species and yeasts in the brine is not desired and may lead to the formation of bloaters.

Sour pickles are prepared from salt stock by removing most of the salt by processing and draining, and by adding vinegar to produce the desired degree of sourness. The acetic acid acts as a preservative.

Sweet pickles may be prepared from soured and drained salt pickles. A syrup is made by dissolving 4 to 10 lbs of sucrose and/or dextrose in 1 gal of vinegar and poured over twice its weight of pickles in a container. Spices are then usually added. Switzer and Fabian (1940), in determining the influence of sugar and acids on the flavors of pickles, found that a combination of 75 per cent sucrose and 25 per cent dextrose was preferred to 100 per cent sucrose alone in a 20° Bé syrup of 20-grain acidity. Wadsworth and Fabian (1939) showed that the use of lactic acid in combination with acetic acid in ratios of 1:4 (0.4 per cent lactic acid and 1.6 per cent acetic acid) to 1:9 yielded best results in the finishing of sweet pickles, processed dill pickles, and other pickle products.

*Pickled Green Olives.*—Selected varieties of Spanish type, green olives are harvested at the optimum stage of maturity, graded, treated with dilute lye to remove a portion of the bitter principle, washed to remove the lye, placed in oak barrels, and brined. The



initial salt concentration of the brine will depend upon the variety of olive being processed and may vary from 5 to 6.25 per cent for Sevillano olives to 10 to 15 per cent for Manzanillo olives (Vaughn *et al.* 1943). The salt concentration becomes stabilized as a result of diffusion and is maintained at 7 to 8 per cent throughout the fermentation for Manzanillo olives, and 6 to 8 per cent for Sevillano olives by the additions of small amounts of salt from time to time.

The barrels containing the brine-treated olives are rolled into fermentation yards, after being filled, and allowed to undergo a fermentation which usually lasts for several months. During this period, the desired brine concentration is maintained and the barrels are kept filled with brine to prevent the growth of aerobic yeasts and molds (Webster, 1949). Sugar, usually glucose, may be added to olives containing insufficient of this ingredient at the rate of 2 lbs per barrel or more. Acids, principally lactic, are produced during the fermentation, along with gas, which helps to maintain anaerobic conditions. The pH value of the pickled olives should be 3.8 to 4.0 and the titratable acidity (as lactic acid) 0.7 to 1.0 g per 100 ml of brine, according to Vaughn and associates.

## ACETIC ACID

Acetic acid ( $\text{CH}_3\text{COOH}$ ) is produced from alcoholic substrates through the oxidizing activities of acetic acid bacteria which are classified in the family *Pseudomonadaceae* in the genera *Acetobacter* and *Bacterium*. Its production by fermentation has been reviewed by Cruess (1948) and by Prescott and Dunn (1949). Its formation in food products, along with lactic acid, has already been referred to.

Acetic acid inhibits the development of many species of bacteria, yeasts, and molds. It is used in making catsup, mayonnaise, pickles, and other products (Prescott and Proctor, 1937), and contributes to the overall preservative action. At one time it was used to prevent the growth of rope-producing bacteria and molds in bread, but it has been replaced largely by the use of propionates and sodium diacetate for this purpose.

## PROPIONIC ACID AND PROPIONATES

Propionic acid ( $\text{CH}_3\text{CH}_2\text{COOH}$ ) is produced by various species of the propionic acid bacteria, which belong to the genus *Propionibacterium*. Its formation during the manufacture of Swiss cheese contributes to the flavor of this cheese. Gas formation by the same propionic acid bacteria produces the characteristic eyes or holes in Swiss cheese (Sanders *et al.* 1950).

Uses of the propionates in the food industry have been discussed by Miller (1940), O'Leary and Kralovec (1941), Harshbarger (1942), Macy (1942), Wolford and Andersen (1945), Olson and Macy (1945), and others. O'Leary and Kralovec (1941) reported that rope formation in bread by *B. mesentericus* could be prevented by the addition of 0.188 per cent of calcium propionate to bread dough at a pH of 5.8 and by a 0.156 per cent concentration of the salt when the pH was 5.6. The inhibitory action of sodium propionate towards microorganisms was greater at lower pH (3.5 to 4.5) values than at higher, according to Wolford and Andersen (1945).

Many of the beneficial uses of the propionates are concerned with applications designed to prevent mold growth, for example in bakery products, malt extracts, soft cheeses, and other products.

*Sodium Propionate.*—This substance, which has the formula,  $\text{CH}_3\text{CH}_2\text{COONa}$ , is a white powder. It is soluble in water.

Sodium propionate (Mycoban) is widely used in the baking industry as an inhibitor of molds and bacteria, particularly the bacillus which causes rope formation in bread (*Bacillus mesentericus*). Ordinarily, 2.5 to 3.5 oz of this compound are used per 100 lbs of flour for white bread and 4 to 5 oz per 100 lbs of flour for dark breads. It is added with the other ingredients at the remix or dough stage in sponge and dough breads and with the other constituents in straight doughs.

In making cakes, sodium propionate is added with the baking powder. Under average conditions, the amounts used per 100 lbs of total batter are 1.5 to 2.5 oz for angel food cake and fruit cake of high fruit content; 2 to 3 oz for cheesecake; 3 to 5 oz. for fruit cake of low fruit content; 5 oz for pound cake; 5 to 7 oz for chocolate cake and 7 oz for devil's food cake.

Under average conditions, 1.5 to 3 oz per 100 lbs of mix may be used for pie filler and 3 oz per 100 lbs of dough for pie crust. Its use is not recommended for cream-filled or custard pies.

When sodium propionate is used in bakery products, an equal weight of sodium chloride is left out of the formulas.

Sodium propionate in 0.2 to 0.4 per cent concentrations is used to prevent molding of malt extracts. It is employed also to impregnate wrappers, caps, and packages in order to reduce the incidence of molds.

The treatment of figs and berries with propionate retards the development of molds, according to Wolford and Andersen (1945). The same investigators showed that the treatment of lima beans and shelled beans helped to maintain their quality between the time of harvesting and processing.

*Calcium Propionate.*— $\text{Ca}(\text{CH}_3\text{CH}_2\text{COO})_2$  is a white powder containing 21.53 per cent calcium, which limits its use to a certain extent. It is soluble in water and slightly soluble in alcohol.

During the manufacture of Swiss cheese, it is formed from lactic acid or lactates as the result of the action of propionic acid producing bacteria. It is thus a normal constituent of Swiss cheese where its presence helps to prevent the growth of molds.

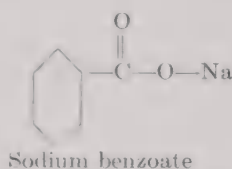
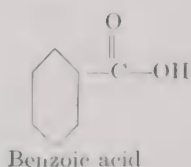
Calcium propionate (Mycoban), like sodium propionate, is used to prevent the development of mold and rope in bread, but is not used extensively in other bakery products. The amounts used for bread are similar to the quantities of sodium propionate employed.

Olson and Macy (1945) have shown that parchment wrappers may be impregnated with propionates to inhibit mold growth on the surface of butter. Impregnated wrappers may be used for other food products.

Propionates may be used to inhibit the growth of molds in chewing and smoking tobacco and in pharmaceutical products.

#### BENZOIC ACID AND BENZOATES

Benzoic acid ( $\text{C}_6\text{H}_5\text{COOH}$ ) and sodium benzoate ( $\text{C}_6\text{H}_5\text{COONa}$ ) have the following structural formulas:



*Benzoic Acid.*—Phenylformic acid or benzenecarboxylic acid occurs in pure form as colorless or white needles or leaflets. The U. S. P. grade is a white crystalline solid or powder; the technical grade is available as flakes, bead-like particles or powder. It is soluble to a limited extent in cold water (1 g in 275 ml). Raising the temperature of the water increases its solubility. Likewise, the incorporation of phosphate and borax increases its solubility in water.

Benzoic acid occurs naturally in cranberries, prunes, green gage plums, cinnamon and ripe cloves; in gum benzoin; and in the balsams of Peru and Tolu.

*Sodium Benzoate.*—This compound is a stable, white granular or crystalline powder, which possesses a sweet and somewhat astringent taste. It is considerably more soluble in water (62.5 g in 100 ml at 25°C) than benzoic acid. For this reason, it is the preferred form for use in many cases.



Sodium benzoate is most effective in acid media and is recommended for use in foods which have a pH of 4.0 or lower. Usually most favorable results are obtained when the pH lies between 2.5 and 4.0 (Cruess and Richert, 1929). If the pH of the product is above 4.0, it may be desirable to adjust it.

Rahn and Conn (1944) reported that benzoic, salicylic and sulfurous acids were nearly 100 times as efficient as antiseptics in strongly acid solutions as in neutral solutions; that only the undissociated acid was antiseptic; that yeast multiplication was inhibited whenever the concentration of undissociated benzoic acid was above 25 mg per ml, and that the toxicity of sodium benzoate (in solution) was due to the undissociated benzoic acid molecule.

Sodium benzoate, or benzoic acid, is employed usually in amounts of 1/10 of 1 per cent or less for the preservation of a rather wide variety of foods, including certain types of carbonated beverages, fountain syrups, apple and other fruit juices, sugar cane juice, relishes, pickles, margarine, jams, preserves, fish, and other items. In addition it is used as a preservative for cosmetic creams, dentifrices, lotions, pharmaceuticals, and textile sizing.

These compounds have been used for the preservation of fish by means of dips or germicidal ices. Such uses have been reviewed by Dunn (1947). The use of benzoate coated wrappers for fish fillets was discussed by Crowther (1940).

The use of sodium and magnesium benzoates and mixtures of benzoic acid and benzoates for treating fillets and possibly round fish was recommended by Fellers and Harvey (1940). The compounds were most effective in a pH range of 4 to 4.5. Concentrations of 0.15 to 0.5 per cent of the benzoates were recommended for use in the dipping solutions. Only a small amount (about 0.01 per cent) of the preservative was retained by the fillets after dipping for 2 minutes in a brine containing 0.3 per cent of sodium benzoate.

Tarr and Sunderland (1938) also found that the keeping quality of fillets (subsequently smoked) was slightly enhanced by immersion in 20 per cent brines containing benzoic acid.

The use of sodium benzoate and other agents to preserve freshly-shucked clams was studied by Piskur and Stansby (1946). Clams stored in 0.1 per cent benzoate in a 3.5 per cent brine possessed a satisfactory storage life of 12 to 13 days when packaged in glass containers and packed in crushed ice. The best treatment consisted in dipping the clams in a benzoate-salt mixture and then storing them in a citrate or phosphate buffer solution at a pH of 5.2 to 5.3 in glass containers held in crushed ice. The flavor and palatability of freshly-shucked clams treated in the foregoing manner was not adversely affected.

Mixtures of benzoic acid and boric acid (not permitted in the United States), or their sodium salts, have been employed in Germany and elsewhere to prevent the spoilage and reddening of salted codfish and to preserve anchovies, caviar, crabs, fish roe, spiced herring and other fish or fish products (Dunn, 1947).

Ices rendered germicidal by the incorporation of benzoic acid (Tarr and Bailey, 1939) or of benzoates (Frandsen, 1946) have value in reducing the bacterial contents of fish.

The use of benzoic acid and benzoates is subject to state and federal regulations which must be complied with. When employed in a food, the package label must contain a statement to this effect citing the amount used.

### SULFUR DIOXIDE

Sulfur dioxide ( $\text{SO}_2$ ) is a colorless, non-inflammable gas with a suffocating odor. When mixed with water, it forms sulfurous acid. It is a reducing agent.

Sulfur dioxide is prepared by the burning of sulfur or by release from sodium sulphite. On contact with water it forms sulfurous acid. The latter may be converted to a sulfate by oxidation.

Sulfur dioxide has a number of uses as a food preservative. It is employed to treat fruits before drying or dehydration, to treat vegetables prior to dehydration, to extend the storage life of fresh grapes, and to prevent the growth of undesirable microorganisms during wine making.

The light-colored tree fruits, such as apples, apricots, figs, peaches, and pears, contain tannin-like substances which are readily oxidized by enzymes when the surfaces have been cut or bruised, giving rise to a brownish discoloration. Sulfur dioxide prevents enzymatic oxidation and apparently also inhibits non-enzymatic discoloration.

With the exception of persimmons, all cut fruits are sulfured before drying in order to help retain quality and light color (Mrak and Phaff, 1947). One practical method of treatment is to expose the fruit halves on trays to the fumes of burning sulfur. The use of sulfur dioxide from cylinders, distributed with a fan, may be substituted for sulfur burning, but it is a more expensive method. Sulfite solutions penetrate the fruit poorly and leach the sugar and are therefore impractical to employ.

The amount of sulfur dioxide added to fruits usually varies from 1,500 to 2,500 parts per million, but may be higher.

Ripe fruit absorbs sulfur dioxide more slowly than unripe or green fruit, but holds it better during and after drying (Phaff and Mrak, 1948).

According to Stadtman *et al.* (1946), the storage life of dried apricots is directly proportional to the initial sulfur dioxide level. The gas disappears during storage at a rate roughly proportional to the logarithm of its concentration. Approximately 65 per cent of the sulfur dioxide initially present is lost during storage.

More detailed information on the sulfuring of fruits is available in publications by Perry *et al.* (1946), Phaff and Mrak (1948), and Mrak and Perry (1948).

Sulfur dioxide is usually applied to vegetables, after blanching and before dehydration, in the form of a sodium sulfite solution. Sufficient sulfur dioxide is added in this manner to provide the amount required. During World War II, cabbage received 750 to 1,500 parts per million; carrots, 500 to 1,000 ppm; and white and sweet potatoes, 200 to 500 ppm (Dunn and Highlands, 1947).

The use of sulfites, or sulfur dioxide, to treat vegetables prior to dehydration aids in the prevention of deteriorative changes during dehydration (reflected in increased storage life), in the preservation of the natural color and flavor of the product, and in the retention of ascorbic acid and provitamin A. The use of excessive amounts of sulfur dioxide results in injury to the flavor and unnecessary destruction of thiamin.

The use of sulfur dioxide to delay the decay of grapes, especially that caused by species of *Botrytis*, is based upon the researches of Winkler and Jacob (1925) and Jacob (1929, 1947). A concentration of about 2 per cent is desired in refrigerator cars or storage rooms for the treatment of grapes. The gas may be supplied from cylinders, by burning sulfur, or by releasing it from sodium acid sulfite. It may be necessary to repeat the treatment several times in cold weather. Likewise, it may be desirable to exhaust the storage area after application of the gas to avoid over-treatment. Jacob (1929, 1947) suggested a level of 15 to 20 ppm of sulfur dioxide in table grapes and one of as high as 50 ppm in wine grapes. The use of too much sulfur dioxide is indicated by its flavor in the grapes, by the appearance of off-flavors or by bleached areas on the fruit.

Sulfur dioxide is extensively used in preparing and preserving wine. It is added to the expressed juice (must) of grapes used for making wine in order to inhibit the development of undesirable types of microorganisms. The concentration used depends upon the source of the sulfur dioxide and the soundness, stage of maturity, and general condition of the grapes before crushing. Amerine and Joslyn (1940) have prescribed the amount of sulfur dioxide to be used under various conditions (refer to table 102).



Sulfur dioxide does not seriously interfere with the action of the wine yeast nor does it impart undesirable flavors to the finished product when used in proper concentrations.

The use of sulfur dioxide in food materials is subject to government regulation.

TABLE 102. — AMOUNT OF SULFUR DIOXIDE TO BE ADDED UNDER VARIOUS CONDITIONS\*

Condition and temperature of grapes	Liquid sulfur dioxide		6 per cent sulfurous acid solution		Potassium metabisulfite	
	Per 1,000 gals of must	Per ton of grapes	Per 1,000 gals of must	Per ton of grapes	Per 1,000 gals of must	Per ton of grapes
	oz	oz	gals	pints	oz	oz
Clean, sound, cool, and underripe	10	2	1½	2	20	3½
Sound, cool, optimum ma- turity	15	2½	2	3	31	5
Moldy, bruised, hot, over- ripe, low in acid	36	6	3½	4½	56	9

\* Amerine, M. A., and Joslyn, M. A., 1951; Table Wines. The Technology of Their Production in California, University of California Press, Berkeley. (Reprinted by permission of the authors and publishers.)

CARBON DIOXIDE

Carbon dioxide (CO<sub>2</sub>), or carbonic acid gas, is colorless, odorless, and non-combustible. One volume of the gas dissolves in 0.6 volume of water at 0°C and in 13 volumes of water at 25°C. It is, thus, more soluble in water at lower temperatures.

Carbon dioxide has a number of rather divergent uses in the food industry. It is employed for the gas storage of fresh fruits, vegetables, and animal products, and for the preservation of fruit juices. It is used in the bottling of soft drinks, beers, and ales. It is used for the replacement of oxygen in cans of dehydrated foods. It has been investigated as a preservative for milk and other dairy products.

Its inhibitory and germicidal action at low concentrations is probably due principally to its effect in reducing the hydrogen-ion concentration of the substrate and in providing conditions unfavorable for the development of some aerobic microorganisms. In high concentration it is believed to act as a protoplasmic poison (McCulloch, 1945 and Schmitthenner, 1949).

In the successful application of gas storage, it is necessary to control both the oxygen and carbon dioxide contents of the atmosphere. In the storage of apples, attention must be paid to the variety being stored, for different varieties require different combinations of carbon dioxide and oxygen. For example, an atmosphere containing about 5 per cent carbon dioxide and 3 per cent

oxygen is best for certain varieties. However, the optimum carbon dioxide concentration may vary from less than 5 per cent to as high as 10 per cent and the oxygen concentration from 2.5 per cent or lower to as high as 21 per cent (von Schelhorn, 1951). A carbon dioxide content of about 2.5 per cent is employed for the refrigerated storage of eggs and one of 10 to 20 per cent for the storage of meat.

As a result of controlling the carbon dioxide and oxygen contents of the storage areas, it is possible to retard both respiration and ripening processes in fruits. Spoilage is thus delayed, for fruit undergoing ripening is less susceptible to microbial decomposition than ripe fruit. Too low an oxygen concentration or too high a carbon dioxide concentration may lead to spoilage of a non-microbial nature.

A high concentration of carbon dioxide may be used successfully when the period of protection is short. Dry ice has been recommended as a source of the gas for the storage of peaches (Kaloyereas, 1949).

Carbon dioxide has been used as a preservative for fruit juices with varying degrees of success. In one process, developed by Bohi (1912), yeast growth is inhibited by the use of 1.5 per cent by weight of carbon dioxide in the juice. However, alcoholic fermentation is not completely suppressed unless the juice contains only a small number of yeast cells. Provided that juices may be prepared to contain only a very small number of cells, the method may be used successfully.

In the beverage field, carbon dioxide is used both for its action as a preservative and as an effervescing agent. The use of carbon dioxide in soft drinks inhibits the development of a number of types of bacteria, including *Salmonella typhosa*, *Corynebacterium diphtheriae*, *Brucella abortus*, and others (Valley and Rettger, 1927) and molds. The development of many microbes is prevented by the low pH (3 or lower) found in some of these beverages.

In the making of beer and ale, the exclusion of oxygen through the use of carbon dioxide and careful production methods prevents deteriorative changes due to oxidation. The anaerobic conditions produced in bottles and cans aid in keeping the product. Hops, alcohol, and heat treatment also contribute to the preservation of malt beverages.

Carbon dioxide is used in the dehydration industry to replace the oxygen of the containers. It was used extensively during World War II in the packing of dehydrated carrots and cabbage. By helping to prevent oxidative changes, it prolongs the life of the product.

The use of carbon dioxide under high pressure increases the storage life of milk, according to Prucha *et al.* (1922).

### SODIUM CHLORIDE

Sodium chloride ( $\text{NaCl}$ ) is widely used as a preservative, alone or in combination with other factors. It may be added to the food in dry form or as a brine, or it may be pumped into the tissues.

Sodium chloride, when used in high concentrations for preservation purposes, produces a high osmotic pressure and causes water (and probably substances soluble in it) to leave the cells of the microorganisms resident on or in the food substance. The loss of water by the cells amounts to dehydration and a condition of plasmolysis results. Microorganisms, in general, fail to grow and reproduce when in solutions of high osmotic pressure. Some microbes will actually be destroyed with the passage of time; others will be inhibited. However, there are some bacteria, known as halophiles, which are able to withstand high concentrations of sodium chloride and may cause eventual spoilage of the salt-treated foods. Tanner (1944) has discussed spoilage by halophiles.

Meats are cured with sodium chloride, usually combined with sodium nitrate and sodium nitrite or potassium nitrate or potassium nitrite, with or without smoking, and with low temperatures. The salt and low temperature are the principal preservative factors during the curing process.

Yesair and Cameron (1942) reported that sodium nitrate, sodium nitrite and sodium chloride showed inhibitory effects on the germination of the spores of *Clostridium botulinum* (No. 62A) in different degrees and that "salt combinations representative of curing practices may result in growth reduction of 100 per cent."

Fish are preserved with salts and smoking also.

The preservation of meat and fish products by the use of salts and smoking has been discussed in detail by Prescott and Proctor (1937), Von Loesecke (1949), Jacobs (1951), Tressler (1951), and others.

### SODIUM NITRITE

Sodium nitrite ( $\text{NaNO}_2$ ) is used as a component of salt mixtures in the curing of meats. In Canada, Tarr and Sunderland (1940 *a, b, c*) and Tarr (1946) have demonstrated that ice containing 0.1 to 0.5 per cent of sodium nitrite is a better fish preservative than ordinary ice or benzoate-containing ice. Promise has been indicated in the use of sodium nitrite in dips for use in preserving fish (Dunn, 1947).



## SUGARS

The sugars used in food manufacture, excepting lactose which is a normal constituent of dairy products, are ordinarily sucrose, glucose, and invert sugar which is a mixture of glucose and fructose.

Sugars perform an important role in the preservation of foods containing high concentrations (40 per cent or greater) of them. Such food materials include jellies, jams, marmalades, preserves, molasses, honey, maple syrup, maple butter, syrups, liquid sugar, condensed milk, candy, grape juice concentrates, and other items.

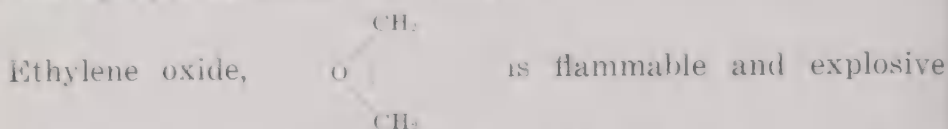
The high osmotic pressure of the sugar creates conditions which are unfavorable for the growth and reproduction of most species of bacteria and yeasts and many of the molds. On relatively rare occasions, bacteria may cause spoilage of chocolate coated candy (Tanner, 1944). A class of yeasts, known as osmophiles or osmotophiles, are able to tolerate the high osmotic pressures found in honey, maple products, syrups, condensed milk, and other foods and may spoil them by fermentation when present in adequate numbers and when unrestricted by chemical or physical inhibitors. Species of *Zygosaccharomyces* in particular, but also *Schizosaccharomyces*, *Torulopsis*, *Saccharomyces* and other yeasts may be involved in the spoilage of honey and other foods of high sugar content (Richter, 1912; Lochhead and Farrell, 1931; Fabian and Hall, 1933; Mrak *et al.*, 1940; Tanner, 1944).

Molds may grow on the surface of syrups, jams, jellies and other saccharine products if these materials become infected after heat treatment and cooling and if a supply of oxygen is available. The effective heat treatment of the closures, the elimination of head spaces, and the usage of the contents of opened containers within a reasonable length of time, reduce the incidence of spoilage caused by this type of infection.

Syrup in storage tanks sometimes becomes infected with osmophilic yeasts and/or molds. The condensation of moisture on the syrup at the top edge of the interior of the tank may reduce the sugar concentration sufficiently to accelerate growth of the microorganisms. The incidence of such infections may be reduced appreciably by the use of a suitable cover over the tank which will keep out dust and microorganisms, the circulation of clean filtered air over the surface of the syrup to discourage condensation of moisture, the use of properly placed and efficient ultraviolet lamps, and the exercise of care in sanitizing or sterilizing the tank before use.

## EPOXIDES

Epoxides are organic compounds in which oxygen is linked to two adjacent carbon atoms in the same chain. Some of them have been described by Whelton and associates (1946). Ethylene oxide and propylene oxide are examples.



when mixed with certain volumes of air. Special precautions must be employed in its use for this reason. Carbon dioxide is occasionally mixed with it in a ratio of 9 to 1 to reduce hazards.

Ethylene oxide has been used in the food industries for a number of years for the treatment of foods and food products. It has been employed to inhibit molds, yeasts and bacteria in packaged dried fruits (usually mixed with isopropyl formate to yield a liquid at ordinary temperatures), according to Whelton and collaborators (1946). It has been used to treat spices and to destroy microorganisms associated with them, especially the thermophilic bacteria (Yesair and Cameron, 1938 and Hall, 1938, 1951). Pappas and Hall (1952) have shown that the numbers of thermophilic bacteria in spices and other food materials may be controlled and greatly reduced by the use of ethylene oxide.

Pappas and Hall (1952) have described a method for treating foods and food materials with undiluted ethylene oxide in which the equipment is grounded and made explosion proof. In this process, the food is subjected to a preliminary heating to a prescribed temperature for 1 hour or longer to aid in the removal of adsorbed and absorbed gases and some of the moisture. Next a high vacuum is applied to the retort room which contains the materials being treated. Then undiluted ethylene oxide is admitted under high vacuum at the rate of about 1.6 lb per 36 cu ft of retort volume and permitted to act upon the heated materials for 2.5 hour or longer. A second vacuum is applied to withdraw the unused ethylene oxide gas, which is diluted with water before disposal in the sewer. Finally the materials are washed with filtered dry air. The entire process requires 6 to 8 hours. Materials may be treated in paper, burlap, cotton, or even multiwall paper bags.

Methods and equipment for sterilization with ethylene oxide have been described by Kaye *et al.* (1952).

## MISCELLANEOUS PRESERVATIVES

*Nitrogen trichloride*,  $\text{NCl}_3$ , may be used effectively to control blue and green and certain other molds which cause spoilage of

some citrus fruits and vegetables (Klotz, 1936; Pryor, 1950). Some data relative to the use of this gas are given in table 103.

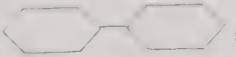
TABLE 103.—DATA RELATING TO USE OF NITROGEN TRICHLORIDE

Fruit or vegetable	Concentration of gas mg per cu ft	Length of application in hours	Frequency of application	Reference
Lemons	1-4	4	3-7 times weekly	Pryor, 1950 Godfrey & Ryall, 1948 Ryall & Godfrey, 1948
Oranges	7	6	1	Littauer, 1947
Cantaloupes	11-25 or 16-23	5 3	1	Barger <i>et al.</i> , 1948 Pryor, 1950
Peppers	4-5	4	2*	Pryor, 1950
Tomatoes	5-12	4	2*	Pryor, 1950

\* On consecutive days

Ozone,  $O_3$ , is a powerful oxidizing agent and unstable. It has been used to retard the spoilage of fruits, vegetables, and other foods. However, there is some question as to its efficacy against microorganisms. Klotz (1936) demonstrated that it was ineffective against the microbes causing decay of citrus fruits. Since it is an oxidizing agent, it accelerates the rancidification of fats. Bacon, bananas, butter, dried eggs, meat, mushrooms and sausages are damaged by concentrations of 50 to 100 ppm (Von Loesecke, 1949). It is toxic to animals and therefore prolonged exposure to it should be avoided.

Borax, sodium borate, in 5 to 8 per cent concentration is used as a 4-minute dip for citrus fruit to reduce decay caused by *Penicillium digitatum* (green mold), *P. italicum* (blue mold), and stem-end rot (Winston, 1935). It is more effective against the former than the latter mold.

Diphenyl,  $C_6H_5 \cdot C_6H_5$  , is used to impregnate wrappers or paper liners for the purpose of inhibiting the organisms causing decay of citrus fruits (Hopkins and Loucks, 1947). The compound may affect flavor adversely, but it is non-toxic to animals in the concentrations ordinarily used (McIntosh, 1945).

Pine oil,  $\alpha$ -terpineol, has disinfecting and deodorizing properties. It is used to treat wrappers employed to prevent decay of citrus fruit caused by blue and green molds. The compound is reputed not to affect the flavor adversely (MacRill and Baum, 1948).

Sodium bicarbonate, baking soda,  $NaHCO_3$ , is used in 2.5 to 3 per cent concentration to reduce the development of green and blue molds and brown rot on citrus fruits (Klotz, 1936).



*Sodium carbonate*, soda ash,  $\text{Na}_2\text{CO}_3$ , in 1.25 per cent concentration is used for the same purposes as sodium bicarbonate.

*Sodium chloroorthophenylphenate* is effective in reducing the development of *P. expansum* (blue mold) and *Botrytis* species (grey mold) on apples and pears (English *et al.* 1948; Kienholz, 1948). It may be applied in 0.4 per cent concentration as a rinse after the removal of the spray residue or in 1.2 per cent concentration with sodium silicate when the latter is used for spray residue removal (Pryor, 1950).

*Sodium diacetate* ( $\text{CH}_3\text{COONa} \cdot \text{CH}_3\text{COOH}$ ) is a bound compound of sodium acetate and acetic acid. It is used commercially (Glabe, 1942) as an inhibitor of molds and rope-forming bacteria in bread. Vinegar, as a source of acetic acid, is satisfactory for controlling rope in bread, but is relatively ineffective against molds when mixed in the dough and tends to impart undesirable flavors to the bread. Sodium acetate has only slight inhibitory effects. The sodium diacetate compound is, however, effective and imparts no off flavors to the bread.

When used in bread, the wrapper should carry a legend such as "sodium diacetate added to retard spoilage" (Kirk, 1953).

*Calcium acid phosphate*.—Monobasic calcium phosphate [ $\text{CaH}_4(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ ] has been used to some extent as a rope inhibitor in bread. It is ineffective as a mold inhibitor when incorporated in the dough.

*Sodium hypochloride*,  $\text{NaOCl}$ , in a concentration of about 6,000 ppm is used to wash citrus fruit (excluding lemons) in an effort to destroy spoilage microorganisms. A concentration of 120 to 140 ppm in refrigerated water reduces the spoilage of peaches (Cardinell and Smith, 1947). Tomatoes, asparagus, and other vegetables may be washed in hypochlorite solutions also (Pryor, 1950).

## ANTIBIOTICS

An antibiotic is a chemical substance produced by a microorganism which possesses inhibitory or germicidal action towards other microorganisms.

A number of antibiotics have been produced commercially, including penicillin, streptomycin, aureomycin, terramycin, chloromycetin, neomycin, bacitracin, polymyxin B, tyrothricin, and others, and are widely used in the field of medicine for the treatment of diseases and infections, particularly those caused by bacteria. Antibiotics are also being used as feed adjuncts and studies are being carried out concerning their possible potential uses for other purposes.

In recent years considerable interest has been indicated in the possible uses of antibiotics in the food field. A preliminary report by Andersen and Michener (1950), concerned with the use of small amounts of subtilin, an antibiotic produced by a strain of *Bacillus subtilis*, with mild heat treatment for the preservation of vegetables in cans, caused a large amount of interest and speculation. Advantages and disadvantages of this method were discussed by Morse (1950). Additional information on the use of antibiotics for this purpose was supplied by Bohrer (1951), Burroughs and Wheaton (1951), Cameron (1951), Cameron and Bohrer (1951), Williams and Campbell (1951), and others.

Foley and Byrne (1950) showed that milk may be temporarily preserved against bacterial spoilage by the use of penicillin.

Adams and associates (1952) studied the effect of subtilin on the spoilage of thermal processed beef, using spores of a *Clostridium* species (P. A. 3679) as the test organism. They found that subtilin inhibited spore germination and that it exerted a "negligible effect on the rate and extent of spoilage" when the concentration of spores was 10,000 per g or greater and the level of the antibiotic was 100 ppm. The presence of 1,000 ppm of subtilin was relatively ineffective in preserving the comminuted beef when combined with mild heat processes of 180° and 212°F.

Godkin and Cathcart (1952) studied the effects of antibiotics in retarding the growth of *Micrococcus pyogenes* var. *aureus* in custard fillings. They found that the growth of this organism and spoilage due to natural heat-resistant bacteria in custard fillings incubated at 37°C were retarded for 48 to 72 hours by the incorporation of 100 ppm of subtilin (70 per cent potency) in the filling. Greater preservative action was secured through the combination of 1.0 ppm of terramycin or aureomycin with 100 ppm of the 70 per cent potency subtilin. Penicillin was less effective than aureomycin or terramycin against the pathogens and demonstrated very slight sporicidal action. In the concentrations employed, bacitracin, chloromycetin and streptomycin were valueless.

English and European fondu cheeses, customarily made from gruyère cheese, are occasionally subject to spoilage by anaerobic spore-forming bacteria. Gas formation by these bacteria may be sufficient to cause bursting of the cans in which the cheese is packed. Proteolysis is associated with gas formation. Nisin, an antibiotic substance produced by *Streptococcus lactis* (strain No. 12), in quantities of 50 units per g of cheese, greatly reduced but did not prevent the growth of the gas-formers. The addition of *Strept. lactis* (strain No. 12) inhibited growth of the anaerobe but was not as effective as the nisin (McClintock *et al*, 1952).

Tarr and associates (1952) studied the experimental preservation of fish and meat with some of the better known antibiotics and found that aureomycin, terramycin, and chloromycetin (in order named) were most effective in inhibiting the growth of the natural mixed bacterial flora at temperatures between 0° and 21°C, and that rimocidin inhibited the growth of yeast. A marked inhibition of spoilage was provided by the incorporation of 0.5 to 2.0  $\gamma$  of aureomycin per g of minced flesh and by immersing steaks in solutions containing 5 to 10  $\gamma$  of aureomycin per ml. Other antibiotics, in concentrations of 10 to 50  $\gamma$  per g, demonstrated either a lower degree of bacteriostatic action or no effect.

Cameron and Bohrer (1951) have listed the requirements of proof which must be established before antibiotics may be accepted for use as food preservatives. In the descending order of essentiality they are as follows: (1) the antibiotic must be capable of destroying the spores of *Clostridium botulinum*; (2) it must be capable of destroying the spores of the saprophytes which cause spoilage; (3) it must be nontoxic and meet other public health requirements; (4) it must satisfy the requirements of food laws and regulations; and (5) it must be practical from an economic point of view.

## CHEMICAL AGENTS USED TO RETARD OR PREVENT SPOILAGE DUE TO OXIDATIVE CHANGES

### GENERAL CONSIDERATIONS

The requirements relating to preservatives used to prevent microbiological spoilage apply in general to the preservatives used to retard or prevent spoilage due to oxidative changes, except that such preservatives must be capable of inhibiting oxidative changes. Government agencies are just as much concerned with the proper use of antioxidants as they are with antimicrobial agents.

There are serious losses in the bakery, dairy, fishery, oil, and meat industries due to the autoxidation of fats and oils with the formation of disagreeable flavors and odors which render the foods unpalatable or inedible. There are losses also in the fruit and vegetable processing industries caused by oxidative changes which lead to discoloration and off-flavors and to reduction in nutritive value.

The chemical substances used to retard or prevent oxidative spoilage in fats and oils are known as antioxidants and are regarded as food preservatives by the Food and Drug Administration and the Bureau of Animal Industry of the United States Department of Agriculture. Substances which enhance the action of antioxidants are called synergists.



Table 104 contains a list of preservatives classed as anti-oxidants or synergists, which the Bureau of Animal Industry (1952) permits to be added "to rendered animal fat or a combination of such fat and vegetable fat" in specified amounts.

TABLE 104. —SOME ANTIOXIDANTS AND SYNERGISTS\*

<i>Antioxidants</i>	<i>Synergists</i>
Resin guaiac	Ascorbic acid
Nordihydroguaiaretic acid	
Tocopherols	Citric acid
Propyl gallate	Lecithin
Thiodipropionic acid	Phosphoric acid
Dilauryl thiodipropionate	Propyl gallate
Distearyl thiodipropionate	
Butylated hydroxyanisole	
Monoisopropyl citrate†	

\* The preservatives listed in this table, with the exception of ascorbic acid, may be added to rendered animal fat or a combination of such fat and vegetable fat, with appropriate declaration and in the amounts indicated in the Regulations Governing the Meat Inspection of the United States Department of Agriculture, June, 1952.

† The use of monoisopropyl citrate is covered in Meat Inspection Division Memorandum No. 179, Aug. 4, 1952.

Space limitations permit only a relatively brief discussion of some of the more important agents used to prevent oxidative changes in foods.

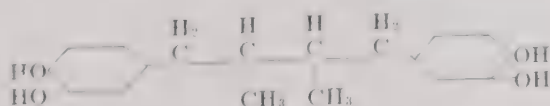
## GUM GUAIAIC

This substance, also known as resin guaiac, is derived from the wood of *Guaiacum officinale* L. or *G. sanctum* L., *Zygophyllaceae*. It contains about 70 per cent of a mixture of  $\alpha$  and  $\beta$  guaiaconic acids, about 10 per cent guaiaretic acid and 15 per cent guaiac yellow, guaiac saponin and vanillin (Merck Index). It is insoluble in water but readily soluble in alcohol and certain other solvents.

Gum guaiac may be added to rendered animal fat or a combination of such fat and vegetable fat in amounts not exceeding 0.1 per cent.

## NORDIHYDROGUAIARETIC ACID

This antioxidant, known as NDGA, 4,4'-(2,3-dimethyltetramethylene)-dipyrocatechol, has the following structure formula:



It is obtained by extraction from *Larrea divaricata*, a desert plant (creosote bush). It is harmless physiologically in the concentrations used. In pure form it imparts no undesirable color, flavor, or odor to lard.

Lundberg *et al.* (1944) described the antioxidant properties of this acid, the use of which is covered in a public service patent by Lauer (1945) assigned to the United States Department of Agriculture.

NDGA is only slightly soluble in fats (about 0.5 per cent by weight at 45°C). It may be incorporated in them by dissolving it first in a solvent, such as alcohol or acetic acid, and then by mixing the solvent with the fat. The solvents are volatilized during subsequent heat treatment. Another method is to dissolve 0.5 per cent of NDGA in hot fat, filter and add the necessary amount of this mixture to the main bulk of the fat to be treated.

NDGA may be added in amounts up to 0.01 per cent to animal fats, especially lard, where it is very effective. Citric acid or phosphoric acid, in concentrations up to 0.005 per cent, act as effective synergists when used in combination with up to 0.01 per cent of NDGA, according to Lundberg and associates (1947). Such combinations are permitted by the Bureau of Animal Industry. NDGA may also be used in combination with butylated hydroxyanisole, with or without citric acid or phosphoric acid.

There is a fair carry-over of the NDGA into baked products made from fats containing it.

## TOCOPHEROLS

There are 4 tocopherols ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) which occur naturally in plants. Wheat germ oil, crude soybean oil, castor bean oil, cottonseed oil, and corn oil are particularly good sources of the tocopherols. The tocopherol content of vegetable oils, fruits and vegetables, cereals and cereal products, animal fats and oils, meats and other products has been reported in detail by Lange (1950). It may be pointed out that vegetable matter is the primary source of the tocopherols in animal tissues.

The tocopherols are components of vitamin E, wherein  $\alpha$ -tocopherol is most active. However,  $\delta$ -tocopherol is most resistant to oxidation, while  $\alpha$ -tocopherol is least resistant.

Tocopherols may be added to rendered animal fat or a combination of such fat and vegetable fat in amounts not exceeding 0.03 per cent. Their activity may be increased by the addition of synergists (Swift and Dollear, 1950-1951). They are fat soluble and insoluble in water. They are relatively stable to heat and therefore are usually carried over into the finished baked products.

## PROPYL GALLATE

This compound is used as an antioxidant or as a synergist for other antioxidants. It is used extensively as an antioxidant for lard and other fats and oils. Such use is covered by patent (Sabalit-schka and Bohn, 1941).

It is only slightly soluble in fats, but is highly soluble in alcohol. It apparently does not produce undesirable changes in color, flavor, or odor in fats.

The toxicity of propyl gallate and antioxidant mixtures containing it has been studied by Orten, Kuyper, and Smith (1948). They found that there was no detectable toxic effect following the prolonged ingestion of this antioxidant or of mixtures when they were ingested in quantities at least 100 times that which human subjects would consume if all of the dietary fat were treated with the effective recommended amounts of propyl gallate or antioxidant mixture.

It may be used in amounts not exceeding 0.01 per cent in rendered animal fat or a combination of such fat and vegetable fat, with or without not more than 0.005 per cent of citric acid. It may be used in combinations containing butylated hydroxyanisole and citric acid.

One oil-soluble combination contains propyl gallate, citric acid, lecithin, and corn oil, and is sold under the trade name of G-4. This combination is readily soluble in oils or melted fats, and is recommended for use in a concentration of 8 oz per 1,000 lbs of lard (Hall, 1950 *a*, *b*, and Sair and Hall, 1951).

## BUTYLATED HYDROXYANISOLE

This compound (BHA), which is essentially a mixture of 2- and 3-tertiary-butyl-4-hydroxyanisoles (Dugan *et al.*, 1950), was announced by the American Meat Institute Foundation in 1948 as a new antioxidant for animal fat.

According to Dugan and associates (1950), the incorporation of BHA, or antioxidant combinations containing it, into lard does not result in the production of detectable flavor or odor in foods prepared with it.

It is particularly effective in delaying the development of rancidity in foods prepared with lard (Kraybill *et al.*, 1948, 1949 *a*, *b*; Van Allsberg, 1950). It is effective alone, or in combination with propyl gallate and citric acid, in retarding rancidification of corn chips, chicken fat, frying oils (corn and peanut), and nuts (Dugan *et al.*, 1950). When incorporated into oils and waxes used to treat wrappers or containers, it aids in preventing the development of rancidity in the fatty films formed therein.



Butylated hydroxyanisole is frequently used in a synergistic combination known as AMIF-72, which contains a mixture of 20 per cent BHA, 6 per cent propyl gallate, 4 per cent citric acid and 70 per cent propylene glycol.\* Sufficient of this combination is employed to produce a concentration of 0.01 per cent BHA, 0.003 per cent propyl gallate, and 0.002 per cent citric acid in the fat, which is usually lard or other animal fat.

Cecil and Woodroof (1951) reported that the shelf life (fresh aroma and flavor) of salted peanuts and pecans was extended by as much as 137 per cent by the use of BHA in the cooking oils and salt. They reported also that the life of the oils was extended about 30 per cent and that the use of synergized BHA increased the oven life of peanut butter to 114 per cent and the shelf life by about one-third after the jars were opened. Treated cooking oils contained 0.02 per cent BHA. The latter was dissolved in a small quantity of oil and mixed with the main batch after it had attained the approximate cooking temperature. Treated salt contained 0.2 per cent BHA. About 1.5 per cent by weight of the salt mixture was added to the nuts, yielding about 0.003 per cent BHA on the surfaces of the nuts.

Sair and Hall (1951) reported that the heating of oils at 350° to 425°F., which are normal cooking temperatures for deep fat frying, rapidly resulted in the oxidation or destruction of BHA, NDGA, and propyl gallate. They demonstrated that success may be achieved on a commercial scale by adding the antioxidant after the cooking process. The antioxidant is mixed with the salt and applied to the hot fried foods immediately after frying, in the cases of potato chips, nuts, or other products which have been deep fat fried. As an alternate procedure for nuts, the authors suggested adding the antioxidant to the dressing oil, which is sometimes used to improve the attractiveness of the product.

Lineweaver and collaborators (1952) have shown that the addition of an antioxidant (Tenox II) to the water used for cooking turkey, in a concentration of 0.005 per cent of the weight of the uncooked turkey halves, markedly retarded the development of rancid flavors and peroxide, both during cooking and during subsequent frozen storage of the creamed product. The antioxidant appeared to be stable under the conditions of cooking (simmering in covered stainless-steel kettles with 1 L of water per 2,270 g of turkey).

According to the Bureau of Animal Industry, U. S. Department of Agriculture (1952) butylated hydroxyanisole and combinations

\*Commercial preparations are available under the trade names of Tenox II and Sustane.

of it with NDGA or propyl gallate, with or without citric acid or phosphoric acid, may be added as preservatives to animal fats and shortenings containing them. Amounts not exceeding 0.02 per cent of BHA, or 0.01 per cent of NDGA plus 0.02 per cent BHA, or 0.01 per cent of propyl gallate plus 0.02 per cent of BHA may be added. Citric acid or phosphoric acid in amounts not exceeding 0.005 per cent may be added with BHA or with combinations of BHA and NDGA or propyl gallate.

#### MONOISOPROPYL CITRATE

This substance may be added to animal fats and shortenings containing animal fats in an amount not to exceed 0.01 per cent (Miller, 1952). However, if it is used with other approved fat preservatives, the amount used is limited to not more than 0.005 per cent.

#### THIODIPROPIONIC ACID

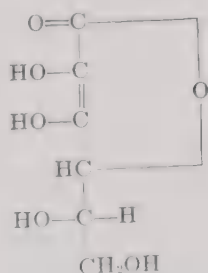
#### DILAURYL THIODIPROPIONATE

#### DISTEARYL THIODIPROPIONATE

These preservatives, or combinations of them, may be added to rendered animal fat, or a combination of such fat and vegetable fat, in amounts not exceeding "0.01 per cent of thiodipropionic acid and 0.09 of either dilauryl thiodipropionate or distearyl thiodipropionate or combinations of the two" (Bureau of Animal Industry, 1952).

#### ASCORBIC ACID

Ascorbic (Vitamin C), which occurs naturally in fruits and vegetables, has the following structural formula:



It is an odorless, white crystalline powder, soluble in water at 25°C to the extent of 34.7 g per 100 ml and insoluble in benzene, ether, chloroform, fats, and oils.

Solutions of ascorbic acid are oxidized when exposed to atmospheric oxygen. Copper, iron, silver, light, and oxidizing enzymes accelerate the oxidation of such solutions.

As a result of basic research by Tressler and DuBois (1944), Fellers *et al.* (1942), Yourga *et al.* (1944), Esselen, Powers and Woodward (1945), and others, *l*-ascorbic acid has been widely used as an antioxidant in the food industry, especially for the treatment of fruits prior to freezing. The acid, alone or combined with citric acid, is effective in preventing the enzymic browning of the cut surfaces of fruits. It adds to the nutritive value of the product with which used and does not impair the flavor when used properly.

Fruit discoloration is due largely to the oxidation of catechols and tannins by the oxygen of the air. The application of ascorbic acid to the cut surfaces of fruits provides an antioxidant which is preferentially oxidized.

Citric acid is used in combination with ascorbic acid for the treatment of certain fruits for it apparently helps to stabilize the latter acid. It has been suggested that the beneficial effect of citric acid may be due possibly to its value in the formation of complexes with iron and copper, to its effect in retarding the action of oxidizing enzymes, and to a decrease of the rate of the oxidation of ascorbic acid.

Strachan and Moyls (1949) found that the treatment of apricots and peaches with 150 to 200 mg of ascorbic acid per 15 oz of finished pack yielded most satisfactory results in respect to odor and flavor retention in frozen sucrose syrup packs. They also found that the best frozen packs of sweet cherries were obtained when using 355 to 637 mg of citric acid and 175 to 250 mg of ascorbic acid per 15 oz of fruit plus syrup. The citric acid enhanced the flavor and color of the cherries.

Ascorbic acid is added to frozen cut fish in order to retard the development of oxidative rancidity during storage. The effectiveness of ascorbic acid in retarding rancidity and rusting or yellowing of fish fillets and steaks has been demonstrated by Tarr (1947, 1948), by Bauernfeind *et al.* (1948), by DuBois (1949), and by others.

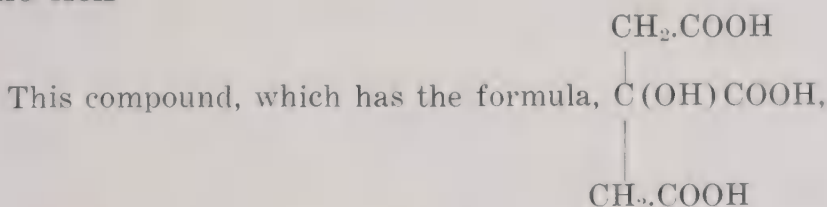
Ascorbic acid may be applied to the fish commercially by dipping or by spraying, according to Bauernfeind, Smith and Siemers (1951). A concentration of 0.5 to 2 per cent of ascorbic acid should be used in aqueous solutions for dipping, or one of 0.25 to 1 per cent if thickened solutions are employed. Carboxymethylcellulose, methyl cellulose and Irish moss have been used as thickeners. The aqueous solution of ascorbic acid should be filtered occasionally to remove accumulated sludge and thereby to reduce bacterial growth. Since



thickened solutions do not lend themselves to filtration, they should be replaced frequently.

Spraying is the recommended form of treatment for large dressed fish. A spray gun, used at low pressure, may be employed to spray unfrozen large dressed fish with thickened ascorbic acid solution or frozen large fish with an aqueous solution of the acid. A minimum of 100 mg of ascorbic acid and a maximum of 200 mg should be used for each pound of fish, according to Bauernfeind *et al.* (1948).

#### CITRIC ACID



is produced in this country largely by the mycological means (Prescott and Dunn, 1949). It is colorless, odorless, and possesses a pleasant sour flavor. It is soluble in water, 1 g dissolving in 0.5 ml.

Citric acid has a number of applications in connection with antioxidants. It is used as a synergist in combination with NDGA, BHA and propyl gallate. It is employed with ascorbic acid to retard the browning of fruits.

DuBois (1949) and others have discussed the use of citric acid with ascorbic acid to prevent the discoloration of cut fruit prior to freezing and during storage. DuBois showed that if citric acid in syrup packs is used in excess of 0.1 to 0.2 per cent of the total weight of the fruit, the latter becomes too tart. Strachan and Moys (1949) found that citric acid in a concentration of 355 to 637 mg per 15 oz of finished product enhanced the color and flavor of sweet cherries. However, they reported that it had no antioxidant value for peaches and apricots when used in amounts permitted edible products.

#### NITROGEN

Nitrogen, an inert gas, is used to replace oxygen in packaged foods and food products and thus aids materially in retarding or preventing oxidative changes. It is preferred to carbon dioxide in the packing of butter and certain other dairy products and citrus fruit juices. Specifically this gas helps to prevent undesirable changes in color, flavor, and vitamin content, particularly carotene and ascorbic acid, and to retard rancidity.

The successful use of nitrogen depends upon several important considerations (Walker, 1951). The oxygen, occluded or dissolved, should be removed from the fresh product before packaging. Likewise, oxygen should be excluded from the package before it is sealed. Packaging materials of low oxygen permeability should be used, which may include gas-tight metal containers or flexible films. Containers or packages should be carefully and adequately sealed by properly operated machines. There should be a good method for testing for possible gas leaks. Finally, packages should be shipped and stored in such a manner as to prevent damage to them. Bayes (1950) also has made recommendations concerning the effective use of nitrogen.

Nitrogen gas is used for the packaging of dehydrated vegetables, dried eggs, dried milk, coffee, dry activated yeast, citrus juices and powder, sandwich meats, sausages, bacon, potato chips, canned sour cherries, baby foods, wines and other products in an effort to extend their storage life through the exclusion of oxygen (Ruff, 1952). Atkinson and Strachan (1950) have described the uses of nitrogen and carbon dioxide gases and ascorbic acid for the preservation of color in apple juice. Nitrogen was preferred to carbon dioxide. Sedky *et al.* (1952) found that the use of nitrogen to displace the oxygen in pliofilm bags resulted in a reduction in the oxidation and discoloration of sauerkraut packaged therein.

## FOOD PRESERVATIVES AND THE LAW

According to the regulations of the Food and Drug Administration nonpoisonous preservatives are permitted to be added to certain foods unless such use serves to conceal damage or inferiority or make the foods appear better or of greater value than is the case.

When a standard of identity has been promulgated for a food under the Federal Food, Drug, and Cosmetic Act, it is unlawful to use a preservative in the food unless the particular standard provides for its use as an optional ingredient (Kirk, 1953). For example, no preservative may be used in catsup; however, sodium benzoate or benzoic acid may be added to jams and jellies.

The food manufacturer should keep himself posted on state and federal regulations, particularly those of the Food and Drug Administration, Federal Security Agency, and of the Bureau of Animal Industry, United States Department of Agriculture.

White (1950) discussed the addition of chemicals to foods with special reference to the oil and fat industry. This article should be of interest to all food manufacturers.

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## 29

# INDUSTRIAL PRESERVATIVES

## INTRODUCTION

IN nature's scheme of things, creation and destruction are simultaneous, continuous processes. By the mechanism of the carbon cycle organic matter is decomposed and resynthesized by biologic agents. With industrial preservatives man attempts selectively to prevent or retard the deterioration of materials of economic value. Man's efforts in this respect date back to ancient times. In the Apocrypha of the Old Testament, it is recorded that Moses addressed Joshua and, referring to the books of the Pentateuch, gave instructions for their preservation by anointing them with cedar oil and storing them in earthen vessels. Probably the first industrial preservative, cedar oil, was favored by the ancients, as indicated by the references to it in the writings of Horace, Ovid, and Pliny the Elder. The remarkable condition of the tombs of the Pharaohs, with their mummies and relics, over thousands of years, serves as proof of the advanced art of preservation as practiced by the Egyptians.

It was not, however, until very recent times—the period of World War II—that the use of industrial preservatives on a mass-production scale was begun. Because of the shortages of almost all types of goods, substitutes had to be made to fill requirements for which they were never intended, and ordinary products and materials had to be treated to make them outlast their expected normal service lives. In addition, replacement of articles which deteriorated when in use by the Armed Services was often difficult, always expensive, and sometimes impossible. But the most serious loss of all was in the lives lost in combat due to rotted tow ropes, fungus-etched telescopes, faulty radio insulation, etc. The Armed Services set up specifications that demanded tropicalization of electronic

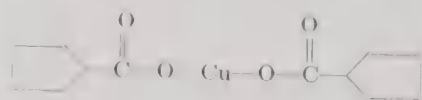


equipment, mildew-proofing of binocular cases and shoes, and rot-proofing of rifle covers, camouflage nets, life preservers, barrage balloons, and numerous other items. Having witnessed the benefits derived from preservative treatment, manufacturers have continued to incorporate these procedures as part of their regular industrial practice.

## COTTON PRESERVATIVES

Next to wood preservatives, those used for cotton assume the greatest economic importance. Mile upon mile of cotton tent cloth is treated to prevent rotting. Tarpaulins, awnings, and tobacco shade cloth are a few of the hundreds of items that receive preservative treatment.

Being cellulosic in composition, cotton is readily deteriorated by cellulose-decomposing bacteria and fungi. Although there is evidence that bacteria may play an important role in the biological deterioration of cotton (Reuszer, 1945), in above-ground service it is generally agreed that the fungi are the most active agents of decomposition. Fungi such as the rapid cellulose fermenter, *Chaetomium globosum* (Thom et al., 1934), and the slow-growing angiocarpous fungi (Zuck and Diehl, 1946) have definitely been shown to be associated with the rotting of cotton fabrics in field exposure although there are many other cellulose-decomposing fungi on the fabric which undoubtedly contribute to the deterioration. Untreated 10-ounce cotton duck lost 67 per cent of its tensile strength in 1 year and 93 per cent in 2 years in shade tests in Florida (Block, 1949). Similar results were obtained in tests in New Orleans (Dean and Worner, 1947) and in the Canal Zone (Barghoorn, 1945). An ideal preservative should have the following properties: It should be high in toxicity to microorganisms, low in toxicity to man, resistant to leaching, stable on weathering, soluble in cheap solvents, colorless and odorless, nonstiffening and nontendering to fabric, noncorrosive to machinery, and inexpensive based upon the cost of the product. The most effective single group of compounds in protecting cotton and other cellulosic textile fibers, such as jute, sisal, and hemp, from microbiological breakdown are the copper fungicides.



Copper naphthenate (one possible structure)

## COPPER NAPHTHENATE

Wheelhorse of industrial preservatives is copper naphthenate. This bluish-green waxy solid with a distinctive naphthenic acid

odor is one of the cheapest, yet most effective, preservatives for cellulosic fibers. It has a low order of toxicity to humans and does not cause dermatitis. It is more widely used than any other textile preservative. Derived from naphthenic acids of petroleum, copper naphthenate is not a pure compound but a mixture of salts of related acids.

The principal sources of naphthenic acid for American production of copper naphthenate are California and Venezuela crude oils. Despite the varied origin of naphthenic acid, copper naphthenate is generally uniform in quality and effectiveness (Marsh *et al.*, 1944). A proposed specification for naphthenic acid to be used in making copper naphthenate is as follows (Minich and Goll, 1946): acid number—not less than 180; iodine value—maximum of 15; flash point—200° F; molecular weight—maximum of 312. For copper naphthenate, the specification calls for no unreacted copper; a copper metal content of 9.2 per cent; a maximum of 3 per cent petroleum ether insolubles; no more than 0.5 per cent water; and a flash point minimum of 200° F. Copper naphthenate has the advantage of being soluble in many common organic solvents, including cheap petroleum oils.

There is considerable evidence of the effectiveness of copper naphthenate. In service tests of copper-treated sandbags, Dean, Strickland, and Berard (1946) rated different copper treatments and found copper naphthenate most effective, on a basis of 1 per cent copper metal. In Florida, copper naphthenate-treated cotton duck, on a basis of 0.1, 0.2, and 0.5 per cent of copper, lost only 39, 24, and 13 per cent, respectively, of its original strength after 2 years' outdoor exposure (Block, 1949). Some preservatives are much more effective in above-ground service than when the material is in contact with or buried in the soil. The copper compounds, and copper naphthenate in particular, maintain their superiority in soil contact and burial. There are many reports in the literature attesting to the efficacy of copper naphthenate on soil-buried cotton (Siu, 1951). Marsh *et al.* (1944) showed that copper naphthenate prevents rotting of cotton fabric in soil at lower concentrations on the fabric than do copper oleate, copper "tallate" or copper hydrogenated resinate. The high preservative capacity of copper naphthenate was related to the fact that naphthenic acid is itself a fungicide. Copper-resistant fungi, such as *Aspergillus niger*, were shown to solubilize and permit leaching of copper from fabric treated with copper oleate and copper "tallate." The fungitoxicity of the naphthenic acid inhibited *Aspergillus* and *Penicillium* organisms and prevented this loss of copper through solubilization. A similar explanation may account for the results of Bayley and

Weatherburn (1947), who reported that, in soil-burial tests of cotton duck treated with copper or zinc naphthenate containing mercury naphthenate or phenylmercuric naphthenate, the mercurials reduced the loss of copper and the breakdown of the fabric. By themselves, the mercurials offer little protection to materials in contact with the soil. A combination of preservatives giving excellent results in actual service tests of rot-proofed sandbags was copper naphthenate plus creosote (Dean *et al.*, 1946). Based on the rating of copper naphthenate alone as 100, copper naphthenate plus creosote rated 176, while the untreated control rated 9.

TABLE 105.—A TYPICAL ROT-PROOF, WATER-REPELLENT, AND FIRE-RETARDANT FINISH FORMULA FOR COTTON FABRICS

	<i>Per cent</i>
42 Per cent Chlorinated Paraffin	26
70 Per cent Chlorinated Paraffin	13
Amberol M-88 (Phenolic Resin)	6.66
Rubbery Pitch (Asphalt)	3.7
Antimony Oxide	20
Calcium Carbonate	12
Copper Naphthenate (as Copper Metal)	0.35
Hydrocarbon Solvent	As needed

Unlike many other preservatives, copper naphthenate does not have to be used with water repellents. In practice, an amount equivalent to 0.6 to 0.8 per cent copper is generally applied to fabric by a bath containing a volatile solvent such as mineral spirits. In military service, fabric may require treatment to render it rot proof, water repellent, and fire retardant. A typical example of such a fabric treatment, the Jeffersonville Quartermaster Depot #242 finish, was used by Barghoorn (1945) in rotting tests in the Canal Zone (see table 105).

There is some question as to whether copper naphthenate accelerates the oxidation of cellulose in the presence of sunlight. It is known that sunlight alone will deteriorate cotton fabric, and by measurement of cuprammonium fluidity it is possible to distinguish between degradation resulting from biological attack and that due to photochemical effects. In the case of biological attack, the fluidity is lower than in the latter case (Dean and Worner, 1947). Copper sulfate appears to accelerate breakdown (Bartlett and Goll, 1945), but there is considerable conflicting literature on copper naphthenate (Weatherburn, 1947). The conclusion expressed by Shanor *et al.* (1945) is that even though a slight loss in the tensile strength of treated cloth might be experienced as a result of copper-accelerated oxidation, the great value of copper



naphthenate in preventing degradation by microorganisms overbalances the other drawback. Further possible drawbacks of copper naphthenate are its color, its odor, and the fact that it stiffens fabric and rope.

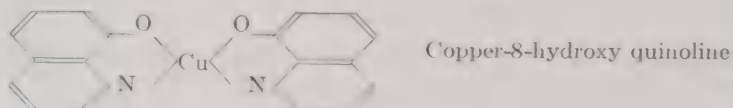
#### COPPER-8-HYDROXY QUINOLINE

Known also as copper-8-quinolinolate, copper oxinate and "copper 8," copper-8-hydroxy quinoline is one of the most interesting and useful of the new preservatives. Although it is many times more costly than copper naphthenate and other common preservatives, its high toxicity to microorganisms combined with other desirable properties have brought it into industrial prominence. On laboratory media, "copper 8" in concentrations of fractions of a part per million will completely prevent the growth of many fungi. It is low in toxicity to humans, does not irritate the skin, and does not stiffen fabric. Its solubility in pure water is 0.8 ppm (Benignus, 1948). It has no odor but has a characteristic yellow-green fluorescent color. It does not tender fabrics in outdoor exposure. In spite of its low solubility in water, it requires formulation with a water repellent for best performance. "Copper 8" is not soluble in common, cheap solvents, and treatment has been accomplished by dispersion in water or organic solvents or by the two bath processes (Monsanto Chemical Company). In the two bath processes, the compound is formed *in situ* on the fabric by first dipping the cloth into a solution of 8-hydroxy quinoline and then into a bath of copper acetate. Fortunately, a process has been developed (Kalberg, 1951 *a, b*) for solubilizing "copper 8" for use in common organic solvents. This is accomplished by heating "copper 8" to 400° F with a metallic soap and a fatty acid. With the solubilized "copper 8" better penetration of the preservative is obtained. Despite its high cost, "copper 8" and the solubilized product are required in many government specifications.

The mechanism by which industrial preservatives exert their antimicrobial effect is not well understood. There has been a lively interest, however, in the mechanism of action of "copper 8." A question arises as to whether the fungitoxicity of "copper 8" is due primarily to the activity of the copper or to the hydroxyquinoline component. Zentmyer (1943, 1944) advanced the "chelation" theory to explain the antifungal action of 8-hydroxy quinoline. Up to that time, it was thought (Hata, 1932) that 8-hydroxy quinoline was acting like another phenolic compound, but intensified by the presence of the quinoline structure.

Zentmyer proposed that 8-hydroxy quinoline precipitated trace metals necessary for cell metabolism and thus the cell was prevented

from growing. Precipitation is accomplished by salt formation through the hydroxyl group and then a chelate ring is formed by the bonding of nitrogen to metal. The metal chelate, such as copper-8-hydroxy quinoline, shown in the formula, is a complex form and



has properties different from those of a simple salt. The chelate properties include decreased ionization, lower water solubility, greater stability, and characteristic color. When Zentmyer added a zinc salt to a fungus inhibited by 8-hydroxy quinoline, the fungus grew, indicating that zinc had been made deficient. Albert *et al.* (1947) reinforced the chelation theory by demonstrating that any modification of the structure of 8-hydroxy quinoline that prevented chelation also destroyed its antibacterial activity. For example, of the seven isomeric mono-hydroxy quinolines only that with the hydroxyl in the 8 position of the ring was capable of chelation and had antibacterial activity. That chelation, *per se*, did not explain the toxicity was shown by tests of many known chelating agents.

Sexton (1949) took issue with the chelation theory of toxicity. He reasoned that if the activity were due to metal chelation, then it might be expected that the metallic complexes, being fully saturated with regard to metal, should have little or no activity. When he found the copper, nickel, cadmium, and silver chelates to be about as toxic as the parent compound, he considered the chelation theory to be dubious. He thought the phenolic theory to be equally as feasible. Mason (1948) tested 8-hydroxy quinoline and its metal chelates with the same results and concluded that the precipitation theory does not completely explain the toxic action. Vicklund and Manowitz (1950) proposed that copper-8-hydroxy quinoline dissociated in solution into copper ions and 8-hydroxy quinoline and that the latter acts by chelation, while the copper may serve to prevent the organism from inactivating the hydroxy quinoline. They offered data on reversal of toxicity with metal ions to support their ideas. Manten *et al.* (1951) explained reversal of toxicity on lowered solubility and concluded that the antifungal activity could not be attributed to precipitation of essential heavy metals.

Rubbo, Albert and Gibson (1950) made the startling discovery that, if all traces of iron and copper were removed from the culture media, 8-hydroxy quinoline was no longer toxic to bacteria. When traces of these metals were added back to the media the toxicity was restored. From this it was clear that the toxicity was due to

the copper and iron chelates and not to 8-hydroxy quinoline. "The toxic effect," they stated, "is undoubtedly initiated by chelation, and is completed by a subsequent poisoning effect of the metal, either in combination with oxine (8-hydroxy quinoline) or as free ferrous ions liberated after transport through the cell wall in the chelated condition. This poisoning apparently takes effect through catalysis of the oxidation of an essential enzyme or metabolite. This destructive oxidation can be prevented by lowering the oxidation-reduction potential." Albert (1951) went on to postulate that by chelation 8-hydroxy quinoline removes a "guardian" metal, like cobalt, which protects an essential thiol group in the cell from oxidation. When the guardian metal has been removed, the iron or copper of the 8-hydroxy quinoline chelate oxidizes the thiol group and prevents normal metabolism.

#### COPPER OLEATE, COPPER STEARATE, COPPER RESINATE, AND COPPER "TALLATE"

These have been used when copper naphthenate was in short supply. These fatty acid and oleo-resinous soaps have been shown to be inferior to copper naphthenate but nevertheless provide adequate protection against cellulose-rotting fungi, if not against surface-growing molds (Goodavage, 1943; Marsh *et al.*, 1944; Tweedie and Bayley, 1944). In other respects they have many of the same advantages and disadvantages of copper naphthenate. They are low in cost and soluble in mineral spirits, but they are blue in color and they stiffen fabric. The fatty acid copper soaps do not have an unpleasant odor. In service tests of sandbags (Dean *et al.*, 1946) copper preservatives rated as follows:

TABLE 106.—THE COMPARATIVE EFFECTIVENESS OF PRESERVATIVES IN PREVENTING THE ROTTING OF SANDBAGS IN ACTUAL SERVICE TESTS

Copper Naphthenate plus Creosote	176
*Cuprammonium plus Creosote	173
Copper Naphthenate	100
*Cuprammonium Carbonate	100
Copper Tallate	82
Copper Oleate	73
Copper Ammonium Fluoride	66
Copper Resinate	45
Untreated	9

\* These were based on 1.5 per cent copper while all the others were on a 1 per cent copper basis.

The copper ammonium complexes, *Cuprammonium Hydroxide*, *Cuprammonium Carbonate*, and *Cuprammonium Fluoride* can be



used where application with an inflammable solvent is undesirable. The basic copper compounds and salts dissolve in aqueous ammonia to give complexes stable in alkaline solution. The fabric is treated by the one-bath process, and ammonia is driven off by heating the cloth to approximately 80° C, thus rendering the treatment resistant to leaching. When proofing with cuprammonium compounds, a range of 1 to 1.5 per cent copper is desirable, which is approximately twice that necessary with copper naphthenate. The comparative effectiveness may be seen in Table 106. Difficulty in application has been encountered, and very close control in treatment is necessary to prevent stiffening and glazing, giving a slippery finish. Dusting out may occur when the fabric is being sewed (Bertolet, 1943). While this process causes a chemical combination between the cellulose fibers and shows good resistance to deterioration by cellulose-destroying fungi, surface-growing molds such as the *Penicillia* are not inhibited (Goodavage, 1943).

#### COPPER HYDROXY NAPHTHENATE

This is not as effective as copper naphthenate, but it has less odor and has been used for that reason and to extend a short supply of naphthenic acid. It is, however, more difficult to apply than copper naphthenate because it is not soluble in hydrocarbon solvents (Shanor *et al.*, 1945).

#### COPPER PENTACHLOROPHENATE

Copper pentachlorophenate gives excellent results in the soil-burial test (Block, 1949), exceeding those with copper naphthenate when compared on the basis of an equal weight of compound. In outdoor weathering for 2 years, copper pentachlorophenate lost only 25 per cent of its initial tensile strength while copper naphthenate lost 39 per cent. Copper pentachlorophenate inhibits not only the cellulose-rotting organisms but the surface-growing molds as well (Hatfield *et al.*, 1944). Disadvantages of the compound are its dark reddish-purple color and its low solubility in common cheap solvents that provide good penetration in a simple one-bath application.

#### COPPER-3-PHENYL SALICYLATE

This is a rather new addition to the family of copper preservatives. Its chief advantages appear to be its lack of odor and its low toxicity (Meyer and Gooch 1952). Pure-culture and soil burial tests show that it discourages cellulose-decomposing fungi and surface molds such as *Aspergillus* and *Trichoderma* (Dow Chemical Company, 1949). The compound has a light tan color.

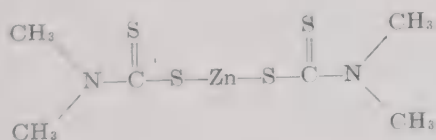
## COPPER CHROMATE

Copper chromate formed on the fabric by a bath of copper sulphate followed by a bath of sodium chromate was found to give excellent protection (Armstrong, 1941). On a basis of equal weight of fungicide, copper chromate contains almost 5 times as much copper metal as copper naphthenate. Of seven copper fungicides compared on an equal weight of fungicide, rather than an equal weight of copper basis, copper chromate gave the best results (Block, 1949). There was a close correlation between the residual amount of copper after two years' weathering and the residual strength of the fabric.

Copper has been employed or tested in combination with cutch, with tannin, with starch, and with humus; but these treatments have been found unsatisfactory or have been superseded for other reasons by preservatives having more desirable characteristics.

## OTHER METALLIC PRESERVATIVES

Other metal and metal-organic preservatives have been evaluated and found inferior to the copper group. An exception may be silver, but silver compounds are too costly to be of practical benefit. There are special applications where copper cannot be used and other preservatives are mandatory. One such application is for textiles in contact with rubber, where copper catalyzes the oxidation of rubber. Another is in camouflage materials, where the high infra-red reflectance of copper defeats the purpose of the camouflage. *Zinc Naphthenate*, while lacking the protective properties of its copper analog, has been used to a considerable extent where a colorless material is necessary. It may also be used in the presence of rubber materials. Furry *et al.* (1941) reported no loss in breaking strength of cotton cloth in laboratory tests with zinc naphthenate. Marsh *et al.* (1944) found that naphthenic acid gave the same protection as zinc naphthenate to soil-buried cotton. It would appear from this that the naphthenic acid rather than the metal is the active component. *Zinc Dimethyl Dithiocarbamate* is an agri-



Zinc dimethyldithiocarbamate

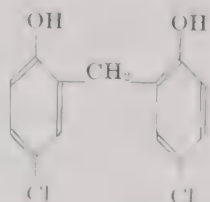
cultural fungicide which has been investigated for use on cotton textiles. It is high in toxicity to fungi, has no color and is non-irritant, but has been reported to be unstable in the presence of acids and solubilized by alkalies (Shanor, 1945). Since it is not

soluble in the solvents commonly employed for fabric treatment, a finely pulverized grade was made available for dispersion in water.

Compounds of mercury have not had wide acceptance as textile preservatives. The most important reason is that copper preservatives are superior and not as dangerous to handle. In addition, mercurials have little preservative effect on cotton in contact with soil. They are much more toxic than copper to the surface molds, or mildew, that stain the fabric and help to leach out the toxic agent, thus contributing indirectly to breakdown. As was mentioned in the discussion of copper naphthenate, mercurials have been found beneficial in combination with copper preservatives on soil-buried cotton. Of the compounds that have been employed for treating fabric *Phenyl Mercuric Triethanolammonium Lactate* is water soluble but resistant to leaching, while *Phenyl Mercuric Oleate* and *Phenyl Mercuric Naphthenate* have solvency in petroleum solvents. They are all colorless.

#### NONMETALLIC PRESERVATIVES

*Dichlorophene*, chemically (2,2'-methylenebis (4-chlorophenol), known also as "Compound G-4," is one of the newer fungicides which made its appearance and gained popularity during the period of World War II. Originally developed, along with its relative,



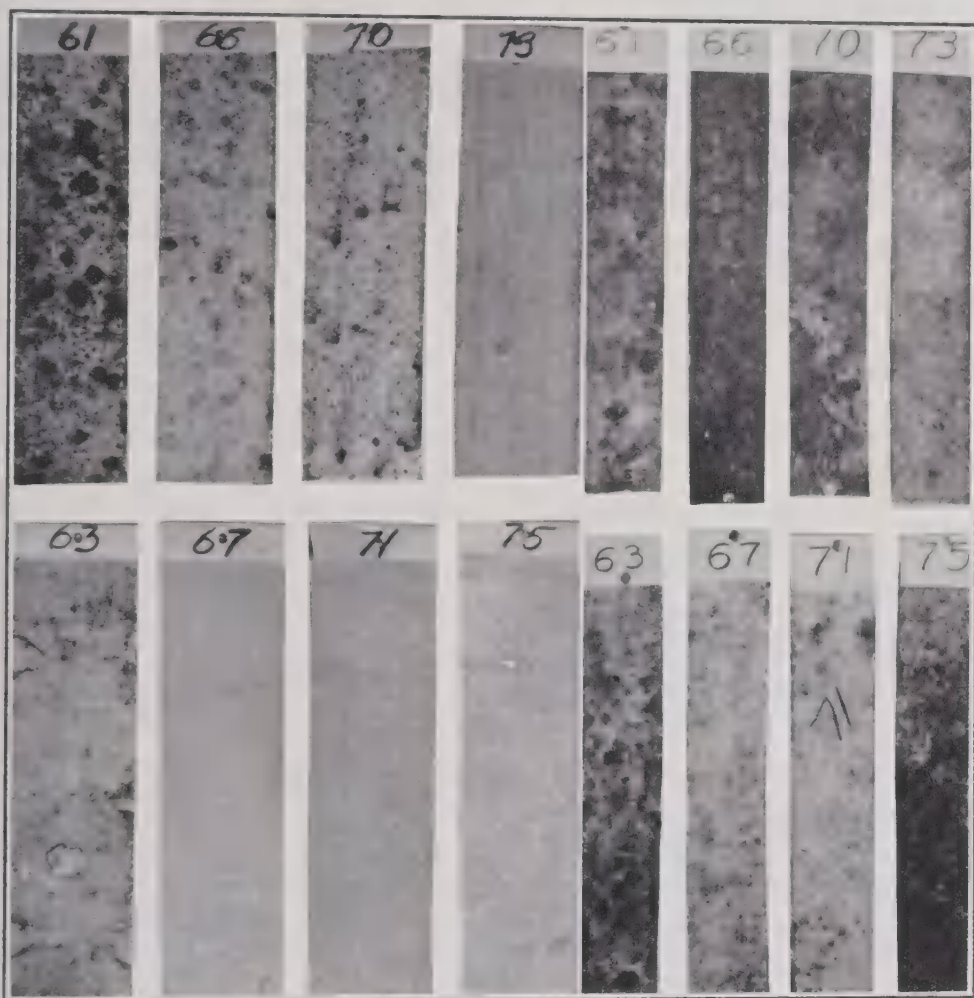
Dichlorophene

hexachlorophene, for the pharmaceutical industry, the compound soon showed its excellent protective properties in tests on cotton fabric. In a report of the Chemical Warfare Service (1945) in which textile fungicides were evaluated on the basis of numerous tests, the highest possible rating was 23 points. One per cent of dichlorophene on the fabric, applied in alcohol, gave a rating of 18 points. The highest rating was 22 points, achieved by a fabric treated with a mixture of copper naphthenate (0.24 per cent copper) and 1 per cent dichlorophene. Marsh and Butler (1946) tested a large number of other bisphenols as cotton fabric preservatives but none was superior to dichlorophene.

In the Canal Zone (Barghoorn, 1945) cotton duck treated with 2 per cent dichlorophene and water repellent showed no loss in



strength after ground contact for 7 weeks and after soil burial for 4 weeks. There was 59 per cent loss after 13 weeks soil burial. Cotton sheeting, similarly treated, showed greater deterioration than the control in sun exposure.



After one year outdoor weather exposure.

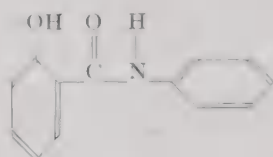
After two years outdoor  
exposure.

Fig. 17.—Effect of Water Repellent on Cotton Strips Treated with Phenolic Preservatives. (Courtesy of Industrial and Engineering Chemistry.)  
Samples at bottom have water repellent treatment, those at top do not.

A condensation product of formaldehyde and p-chlorophenol, dichlorophene has only a slight antiseptic odor and adds no color to the fabric. It is very insoluble in water but may be applied to cloth by first dissolving it in an alcohol and diluting this solution with a petroleum solvent. As with other similar preservatives, a

water repellent is desirable to help prevent leaching. The compound is nonirritating to the skin, has low toxicity, and is active against both mildew and rot fungi.

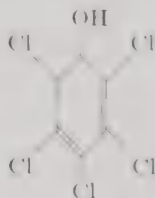
*Salicylanilide* or "Shirlan" has given satisfactory service in textiles for over 25 years. It was especially developed for protection of textiles by workers of the British Cotton Industry Research Association. Selected from many compounds, salicylanilide, the reaction product of aniline and salicylic acid, was found to have the best combination of desirable properties (Fargher *et al.*, 1930). It is effective against both roppers and mildews, has practically no odor, does not color fabrics, and is not hazardous to use. Like other phenolic compounds, it is susceptible to leaching in outdoor weathering and should be used with a water repellent, as shown in Figure 17.



Salicylanilide

Application to cloth is effected by emulsions or dispersions or as the ammonium salt. In the latter process, ammonia is driven off in the drying, leaving a water-insoluble residue.

*Pentachlorophenol* is a powerful fungicide which has come into wide application in wood preservation. It has been used in textile treatment but has certain objectionable characteristics. It is a primary skin irritant and though low in water solubility is leached rather rapidly from the cloth. It has been said to decompose in sunlight giving hydrochloric acid, which tenders the fibers, but that has not been proven (Bertolet, 1943). Pentachlorophenol



Pentachlorophenol

has been successfully employed for camouflage fabrics. In this application rot prevention is not required since the fabric serves only to give texture and color to a net. The preservative is required to prevent growth of superficial molds and not affect the durability of the colors (Shanor, 1945). The low cost of this compound has

attracted wide interest, and it has been employed on fabrics in storage and other applications where it is not subject to weathering. A quaternary ammonium pentachlorophenate, *Trimethyloctadecyl ammonium pentachlorophenate* was found to retain much of the fungitoxic activity of pentachlorophenol without the irritating properties. This compound was used to treat thread, but was found to deteriorate in sunlight.

*Mercaptobenzothiazole* is an effective organic fungicide, toxic to superficial molds and cellulose-decomposing fungi. It is useful as a mildew preventive although it has a somewhat unpleasant odor in the concentrated form. Application may be made from



2-Mercaptobenzothiazole

alcohol or by preparing a concentrate in cellosolve which may be diluted with volatile petroleum solvents. More recently this compound has been used in combination with other fungicides.

*Acetylated Cellulose*, while not a preservative of itself, is the result of a preservative treatment, the acetylation of cotton. Acetylation is only one example of a general approach to the preservation problem, namely, modifying the cellulose to make it nonsusceptible to the enzyme systems of microorganisms and thereby immune to biological deterioration. A discussion of this approach (Siu 1951) shows that it has produced results. The extent to which modification processes will be used is largely dependent on their ability to justify the increased cost over that of the simpler treatments with microbiological inhibitors.

## WOOL PRESERVATIVES

Wool is a protein fiber and is much more resistant to attack by fungi than is cotton. It can be deteriorated by the "athlete's foot" fungus, *Trichophyton interdigitale*, as has been shown by Rogers *et al.* (1940); but deterioration, when it does occur, is generally brought about by proteolytic bacteria such as *Bacillus mesentericus*, *Bacillus subtilis*, and *Bacillus fusiformis* (Burgess, 1924). Inasmuch as bacteria require much higher quantities of moisture than fungi, there is little deterioration of wool unless it is wet. Soil-buried wool is quickly decomposed, as is soil-buried cotton. From a microbiological standpoint the important economic problem in wool



preservation is the prevention of the growth of surface molds which do not decompose the wool but which spot and stain it and frequently make it unmarketable. Mildew or bacterial infection may also result in uneven dyeing of the wool. Pure, scrubbed wool is practically nonsusceptible to mildew, but there is usually sufficient organic matter in the form of oils, soaps, conditioning agents, and dust in the wool to support mold growth (Burgess, 1929). Wool is more hygroscopic than cotton and will mildew in an atmosphere of lower relative humidity, for, as has been shown (Block, 1953a), the higher the equilibrium moisture content of a material the lower the relative humidity at which it will mildew, other things being equal. Chemical degradation of wool and increased water solubility, as may be caused by ultraviolet radiation, will increase the susceptibility to mildew (Burgess, 1934).

Burgess (1924, 1928, 1930, 1931, 1934) made a thorough study of the problems associated with the growth of microorganisms in wool. He tested 150 preservatives and found only sodium fluoride, sodium fluosilicate, and salicylanilide to be satisfactory. Beta naphthol gave good results, but it was found that if mildew did occur there was a dye reaction with the products of the fungus and the cloth was stained. Sodium fluosilicate was not as effective against molds as salicylanilide but was more active as a bactericide. In addition, it imparted some moth-repelling properties to the wool. A number of common wool dyestuffs were tested but did not have disinfectant action. Formalin was unsatisfactory as a growth inhibitor, phenol was no good under 8 per cent, mercuric chloride at 2.5 per cent stopped bacterial growth but not molds, and it also yellowed the fabric. Two per cent of salicylic acid or hydrogen peroxide did prevent mildew or tendering. Chromium in a concentration of 1 per cent prevents mildew. Bayley and Weatherburn (1945) recommend the use of 1 per cent of potassium dichromate on the goods as a preservative for woolen blankets, sleeping bags, and socks for use in the jungle. As used in the dyeing of wool, chromium gives resistance to mildew, but it apparently acts by removing the degradation products which support mold growth and not as an antiseptic, for the concentration of potassium dichromate is insufficient to stop mildew.

Deterioration of wool by microorganisms is serious in storage and shipment and in the processing mills. For the first problem, Burgess (1931) recommended ventilation of the storage containers and the holds of the ships in addition to a preservative treatment with salicylanilide. In the mill, fungus is more difficult to control than bacterial growth, for the latter is no problem if the water is kept slightly acid. Preservatives may be added at several stages

of the processing of wool in the plant (Burgess, 1931). In the winding process, 2 per cent of salicylanilide may be applied with the water for conditioning the cloth. A more uniform treatment can be obtained in the backwashing process, but the preservative must not react with the soap. Sodium fluosilicate could not be applied in this step because of its reaction with the soap. In mill runs, 0.6 per cent of salicylanilide and 0.67 per cent of the sodium salt of o-phenyl phenol were satisfactory. During the oiling process, 0.2 per cent of salicylanilide on the wool, applied in the olive oil-water mixture, gave protection over 22 days. Volatile fungicides such as p-chloro-m-cresol were not satisfactory. Slight color changes of light shades of wool were observed when wool was stored in closed containers at high humidities with various volatile phenolic and camphor substances. Undyed wool was kept free of mildew and in good condition at 100 per cent humidity for 2 years in a closed container with p-dichlorobenzene crystals (Block, 1951).

## LEATHER PRESERVATIVES

Leather, like wool, is a proteinaceous material. Its susceptibility to attack by microorganisms in some ways resembles that of wool and in some ways differs. The differences result in part from the unique processing that leather undergoes. This includes salting, pickling, bating, tanning, and finishing. Before it is tanned, skin for leather may be rotted by bacteria, but the finished leather is not rotted even in soil burial (Kanagy *et al.*, 1946). When army shoes fell apart in the New Guinea Campaign (Anon, 1942), the difficulty was traced to deterioration of the cotton stitching rather than damage to the leather itself (Abrams, 1948). The same superficial mold flora that contaminates wool grows on leather. Most common are the *Penicillia* and *Aspergilli*. From a sample of leather goods returned from the South Pacific, Dr. Seth Pope of the U. S. Department of Agriculture isolated 9 species of *Penicillium*, 2 species of *Aspergillus*, and 1 species each of *Mucor* and *Spicaria* (Army-Navy National Defense Committee, 1944). While mildew does not cause any appreciable deterioration of the hide substance in leather, there may be an increase in stiffness and loss in tensile strength. The grain also is weakened and tends to crack, according to tests made at the Bureau of Standards (Kanagy *et al.*, 1946). It has been demonstrated that, in addition to the water-soluble constituents, the fatty oils and particularly the stuffing grease make finished leather susceptible to mildew (Abrams, 1948). The deleterious changes in the physical properties of leather after mold growth have been attributed, at least in part, to the removal of the grease.

Much work has been done on preservatives for leather. For the finished leather, the organic mercurials successfully control mildew when employed in very low concentration. Colin-Russ (1940) reported 0.00175 per cent of phenylmercuric nitrate was effective. Concentrations of 0.05 to 0.1 per cent of phenylmercuric stearate, phenylmercuric oleate, or phenylmercuric 2-ethyl hexoate prevented mold growth on leather at 100 per cent relative humidity, whereas 0.5 per cent of the best nonmercury fungicide, pentachloro-



Fig. 18.—Treated and untreated shoes of a pair after storage for one month in a home closet during the summer in Florida.

phenol, was required under the same conditions (Block, 1953*b*). Cordon *et al.* (1949) found 0.03 per cent of phenylmercuric acetate or phenylmercuric lactate complex to be effective, as was also 0.2 per cent of a phenylmercuric octadecanoic complex and 3 per cent of pentachlorophenol. A commercial preparation for use in all stages of leather processing employs phenylmercuric acetate in combination with salts of the chlorophenols (Buckman Laboratories, 1949). Lollar (1944) found 0.5 per cent concentration or higher was required with several organic mercurials, although some of those which were effective at 0.5 per cent were not tested at lower concentrations to determine the minimum effective concentration. The mercurials have not been used very extensively because they may cause dermatitis on prolonged skin contact, as shown by Lollar (1944) for leather bearing 0.75 per cent of three mercurials.

*Paranitrophenol* is the compound workers have found most suitable as a leather antiseptic, and consequently it has been em-



ployed more extensively than any other material for this purpose. Jordan (1934) reported p-nitrophenol more effective than beta naphthol in preventing mildew. For chrome-tanned leather, which requires less preservative than vegetable-tanned leather, 0.1 per cent p-nitrophenol or a similar concentration of chlorophenol salts were satisfactory (Richardson, 1940). Colin-Russ (1940) also found p-nitrophenol to be an efficient leather antiseptic. Based on tests of 40 selected fungicides, Lollar (1944) recommended 0.5 per cent of p-nitrophenol or p-chloro-m-xylene in commercial leathers where maximum resistance to molds is desired. In patch tests for dermatitis, he found no irritation with 0.75 per cent p-nitrophenol in 48

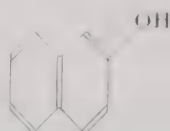


Paranitrophenol

hours. Reporting on tests of fungicide-treated leather conducted cooperatively at four different laboratories, Kanagy *et al.* (1948) concluded that p-nitrophenol and pentachlorophenol were most effective. No mercurials were included in these tests. Pentachlorophenol was even more fungitoxic than p-nitrophenol (0.25 per cent compared to 0.35 per cent for protection) but was not favored because of its tendency to cause skin irritation. With a combination of p-nitrophenol and pentachlorophenol, however, the quantity of pentachlorophenol and p-nitrophenol may be cut to 0.1 per cent each, and the leather dressing will control the growth of *A. niger* (Abrams, 1948). At this concentration of 0.2 per cent total fungicides the leather may be used on articles that come in contact with the body. This fungicidal composition, developed at the National Bureau of Standards, was the basis for a mixture for use on leathers supplied to soldiers in the tropical regions during the latter part of World War II. Except in the case of white leathers, where other compounds must be substituted, the yellow color of p-nitrophenol is no drawback because it is masked by the natural color of the leather. Although p-nitrophenol is fairly soluble in water, it has been shown to be effective on leather even after vigorous washing of the treated leather in distilled water.

Second to p-nitrophenol in order of preference, the British Leather Manufacturers Research Association (1945) named *Beta Naphthol*. It is an old-line antiseptic and fungicide which has been recommended for leather by many workers. Orthman and Higby (1929) found beta naphthol to be both economical and effective in

keeping molds from growing on hides during tanning and dyeing. Colin-Russ (1940) listed it favorably. Abrams (1948) found that after leaching it took 0.82 per cent beta naphthol to eliminate *A. niger*, compared to 0.65 per cent for p-nitrophenol. Other compounds that have been recommended include *Para-Chloro-Meta-*



Beta naphthol

*Xylenol* and *Salicylanilide* (Lollar, 1944), *Tetrachlorophenol* (Greene and Lollar, 1944), *2-methyl-1, 4-Naphthoquinone* (Cordon *et al.*, 1949), and *Cresylic Acid* (British Leather Manufacturers Association, 1945).

Preservatives are not only important for the finished leather but are employed in many stages of leather processing, where skins, hides, and some tanning materials may be damaged by microorganisms. Mold may grow on dried hides and skins, and fungicides have been incorporated in salt used in curing (Stuart and Frey, 1934). During the soaking process, skins and hides may be damaged by bacteria and molds giving offensive odor, excessive hair slippage, and some destruction of the collagen fibers (Buckman Laboratories, 1949). Although pickling is designed to preserve skins, in this process mold and yeasts may stain and damage skins. If pickling is effected at a sufficiently high acidity, pH 2.4, with the proper quantity of sodium chloride, and preferably with acetic acid, mold growth is prevented (Pleass, 1935). Acetic acid was better for this purpose than formic, salicylic, or benzoic acids. Vegetable tanning liquors are subject to attack by bacteria, yeasts, and molds. Of these, the molds are the most important, for certain molds, as *A. niger* and certain of the *Penicillia*, produce an enzyme, tannase, which destroys tannin and causes the tanner to suffer economic loss (Richardson, 1941). A selective disinfectant is desirable to prevent mold and yeast growth but to permit the lactic acid bacteria to continue their desired fermentative activities. Richardson (1941) could not draw definite conclusions from his experiments, but there was some indication that at certain critical concentrations some of the chlorinated phenols inhibit mold and yeast growth to a greater extent than they do bacteria. Salicylanilide and 8-hydroxyquinoline would appear to be ideally suited for this purpose. The British Leather Manufacturers Association (1945) has recommended treatments for use at different stages of leather processing and has given specific directions.

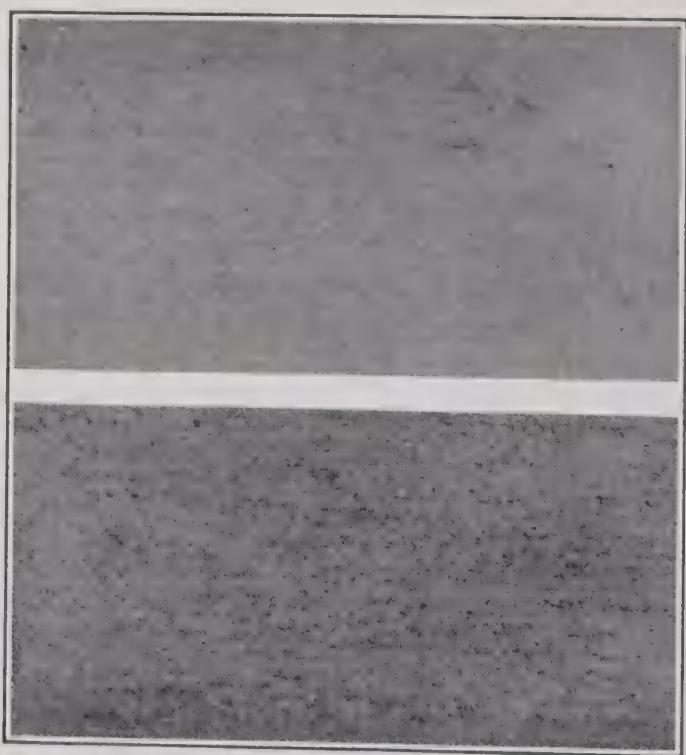
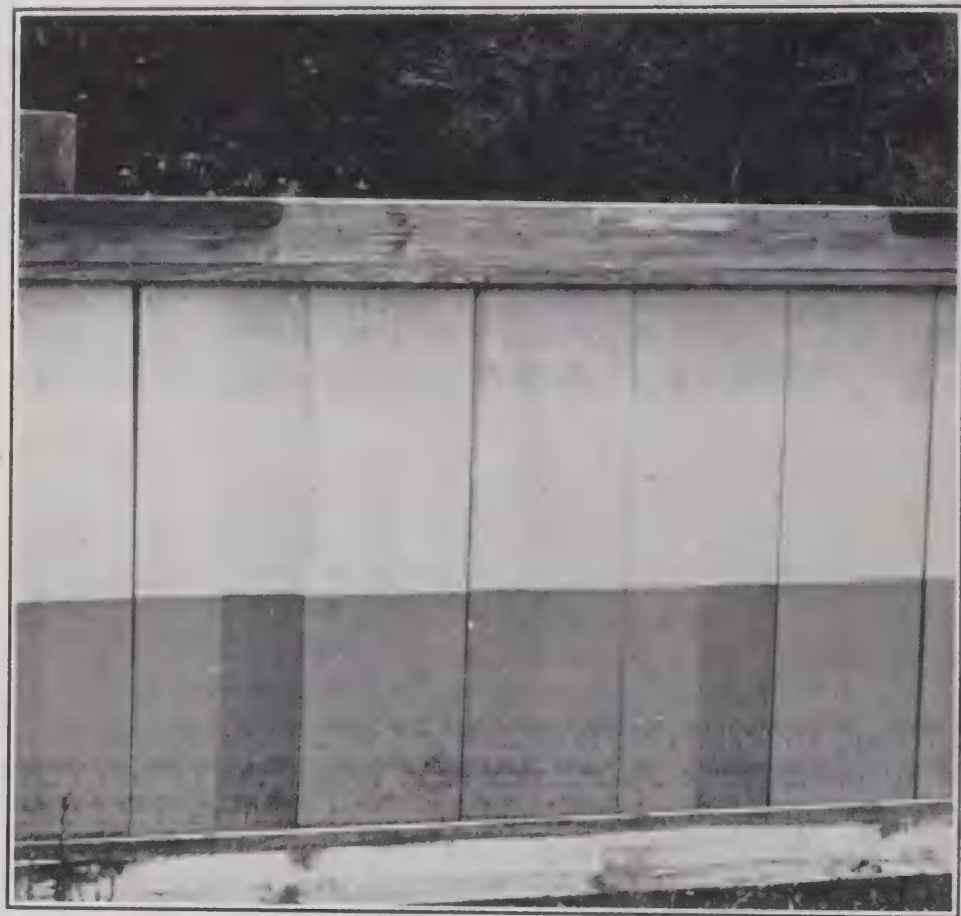
For sterilization, cleaning, and reconditioning of used army shoes, Greene (1945) recommended treatment with aqueous formaldehyde followed by a wash with soap and a treatment with an oil containing pentachlorophenol. Greene and Lollar (1944) developed a formula for an army dubbing containing for its preservative 0.8 per cent each of p-nitrophenol, p-chloro-m-xyleneol, and tetrachlorophenol. Kanagy *et al.* (1948) give a formula for a fungicidal leather dressing employing p-nitrophenol which was adopted by the Office of the Quartermaster General.

## PAINT PRESERVATIVES

Preservatives are used in paints for various purposes. In protein-based water paints they are required to prevent decomposition by protein-decomposing bacteria, with resulting gas formation that may cause blowing-up of the cans. The paint film after application may be susceptible to mildew and should be preserved. Anti-fouling paints for ship bottoms must retard the growth of the many types of microbiological and macrobiological plant and animal forms in order to keep the hulls as free as possible from fouling organisms. For breweries, packing houses, and hospitals, antiseptic paints are desirable. Lacquers and other coatings for electronic equipment should be inhibitory or inert to fungi which, in growth, produce moisture which causes electrical leakage and faulty operation of the equipment.

Mildew of painted surfaces has received considerable attention. The reason is self-evident. A home painted white may be grey and dingy in appearance in 6 months to 1 year under conditions favorable to mildew, and the cost of repainting is many times greater than the cost of the paint itself. The composition of the paint to a great extent determines its susceptibility to mildew. Linseed oil and its oxidation product, linoxyn, serve as a source of carbon for fungi, while residues of mucins or foots, not entirely removed in the refining process, serve as a source of nitrogen (Weise, 1934). Substitution of inert resins for part of the vegetable oil increases resistance to mildew. Modification of the oil by bodying with heat also increases mildew resistance. In general, it can be said that a quick-drying, hard film will be more resistant to mildew than a tacky, soft film. The hard film not only catches less dirt and fewer mold spores but also has a lower moisture content under equal conditions of humidity (Harnden, 1945). Barn paints and green and brown trim paints are examples of films which remain relatively soft and are especially liable to mold attack.





Figs. 19 and 20.—Weather exposure tests of fungicide-treated paint. Closeup photograph shows the appearance of panels painted with treated and untreated paints.

## ZINC OXIDE

Perhaps the most effective mold inhibitor for paint is zinc oxide (Gardner *et al.*, 1934a; Vannoy, 1948). This compound is not specifically a preservative but is a normal component of the pigment formulation of paint. Gardner *et al.* (1934a) reported that white lead paint formulations containing zinc oxide always showed a very marked inhibition of mildew while those without zinc oxide did not. The quantity of zinc oxide is important, for in tests extending over 5 years, Vannoy (1948) showed that paint with only 30 per cent of the pigment as zinc oxide became badly mildewed whereas paint with 50 per cent zinc oxide had no mildew. Harnden (1945) stated that zinc pigments are poor mildewcides but that zinc oxide is effective because of its property of hardening the paint film. Salvin (1944), on the other hand, demonstrated that zinc oxide inhibits respiration of fungus spores and prevents mycelial growth, although it does not kill the fungus. Gardner *et al.* (1934a) attributed effectiveness to both the physical effect and biological activity. Other zinc pigments do not have this property of inhibiting mildew in a paint film.

## MERCURIALS

Mercurials have been shown to retard mildew in paints when used in very low concentration. After years of testing preservatives for paint, Gardner and coworkers (1933, 1934a, 1934b, 1935, 1936, 1937 a, b, 1938 a, b, 1939 a, b) concluded that the mercurials were the best for outside paint. He recommended (1938a) the use of one part of mercuric chloride in 500 to 900 parts of paint, depending upon the severity of the mildew infection. Mercuric naphthenate and phenylmercuric linoleate at a concentration of 1:200 were as effective as mercuric chloride at a concentration of 1:500 in paint. Since the mercuric naphthenate contained only 25 per cent of mercury, the naphthenate was more effective on a basis of equal concentration of mercury. Oil-soluble organic mercurials are preferred to inorganic forms of mercury because they dissolve in the paint, they are not readily leached out by rain, and they are less hazardous to use. Minich and Goll (1946) claim that the phenylmercurials are much more efficient as mildewproofers, and offer toxicological data for their safety in paint. Vannoy (1948) compared phenylmercuric oleate with mercuric chloride and mercurous chloride in paint and found that phenylmercuric oleate gave, by far, the best results on an equal-weight basis. It is interesting to note that, whereas the mercurials are remarkably effective in low concentrations, they are

not correspondingly more efficient with increasing concentration. Organic mercurials have been found to be useful in varnish and lacquer employed for fungusproofing electronic equipment to be used in the tropics. In a statistical analysis of performance tests, Leonard and Pitman (1951) found that 7 out of 10 of the most effective compounds in preventing fungus growth on coated cotton-braid wires in a tropical jungle were organic mercurials. The compounds, in a descending order of effectiveness, were: o-hydroxy-phenylmercuric chloride, salicylanilide, pyridylmercuric chloride, p-toluene sulfonylamide, uranyl nitrate, p-aminophenylmercuric acetate, phenylmercuric-o-benzoic sulfimide, p-acetylaminophenylmercuric acetate, hydroxymercurisalicylic acid anhydride, and phenylmercuric phthalate. As noted by Benignus (1947), varnishes deactivate fungicides, either chemically or by sealing them in the film. In the tests of Leonard and Pitman (1951) even 10 per cent of the compounds in varnish or lacquer did not completely prevent mold growth.

#### CHLOROPHENOLS

Tetrachlorophenol and pentachlorophenol have been employed with success as mildew inhibitors for paint. Gardner (1938a) recommends their use for interior paints where it may not be desirable to use mercurials. For oil paints, Benignus (1947) noted that 2 to 5 per cent of pentachlorophenol is required but that it may change the drying properties of the paint. In casein and other protein-type water paints, 0.5 to 1.0 per cent of the sodium salt of pentachlorophenol serves as a bactericide, as well as a fungus inhibitor. Two per cent was employed in camouflage paints during the war. Benignus was enthusiastic about the zinc salt of pentachlorophenol used as 3 to 5 per cent of the paint and ground in the paint as a white pigment. Tests by Vannoy (1948) on zinc pentachlorophenate and zinc tetrachlorophenate showed that these compounds had merit when employed in high concentrations. Under severe conditions of testing, Partansky (1942) reported that 3 per cent of tetrachlorophenol or its zinc salt preserved both an oil and cold-cut resin type of interior paint for 2 years. Minich and Goll (1946), however, stated that, in tests of paints in a brewery and textile mill, tetrachlorophenol failed completely whereas phenylmercurials provided the paint with immunity against mildew. The concentrations of tetrachlorophenol employed, however, were not mentioned in the latter paper.

#### CUPROUS OXIDE

Red cuprous oxide in a concentration of 5 per cent or greater may be used with very effective results in colored paints, like reds



and browns and some other shades. It is a very efficient material for use as a mildew inhibitor for dark colors, according to Hart and Gardner (1940). In a concentration of 2 per cent, Vannoy (1948) found cuprous oxide to give better retardation of mildew than any other protectant after 5 years exposure. Because of its color it could not be used in light colored paints unless it were found effective in lower concentrations. Vannoy reported such tests in progress. In antifouling paints for ships' hulls cuprous oxide is generally used in combination with mercuric oxide. Since it is not as toxic to the marine organisms as the mercury compound, twice as much copper as mercury is used (Phelps and Dorato, 1943).

#### COPPER-8-HYDROXY QUINOLINE

The only compound to rival the effectiveness of the mercurials in preventing mildewing of paints is copper-8-hydroxy quinoline. In laboratory tests employing an iron oxide-linseed oil paint, Vicklund and Manowitz (1951) found "copper 8" to be superior to mercuric chloride, phenylmercuric salicylate, tetrachlorophenol, and a number of other compounds. One-half per cent of "copper 8" in the paint was sufficient to prevent growth. Richardson and Del Giudice (1952*a*) tested "copper 8" in commercial paints, with the solubilized product giving the best results. When tested under practical conditions of use in food-processing plants (Richardson and Del Giudice, 1952*b*) approximately 1 per cent of "copper 8" as the solubilized product in paint kept the ceiling of a sausage cooler room practically free of mildew for a year while the paint without "copper 8" was completely covered with mold in 3 months. Similar results were obtained in a cook room of a cheese plant. The oil solubility of solubilized "copper 8," as well as its low toxicity and resistance to leaching, combined with its high effectiveness against molds (Herman and Reed, 1952), makes this an ideal toxicant for use in paints for food plants where both humidity and organic nutrients for fungi may be great. Herman and Reed (1952) noted that a cheese plant with unusually severe mold growth on the walls and ceiling required a complete cleanup and paint job 4 times a year, whereas when paint containing solubilized "copper 8" was applied, the problem did not recur for 18 months, and perhaps longer. "Copper 8" cannot be used where a white color is essential, for the compound gives a greenish tint. Though "copper 8" paints are more effective against mildew when used with zinc oxide (Vicklund and Manowitz, 1951), this pigment may tend to produce an uneven coloration known as "livering" (Eisenschiml and Kalberg, 1948). Solubilized "copper 8" was unusually success-

ful in controlling mold growth on vinyl raincoating and shower curtains where plasticizers that serve as food for fungi are employed. "Copper 8" has also been shown to control mold growth in lacquers, varnishes, and waxes used on electronic equipment (Benignus, 1947; Eisenschiml and Kalberg, 1948).

#### MISCELLANEOUS

Gardner (1938a) recommended the use of *thymol* with additional paint drier in interior paints that are to be applied in bakeries, tobacco factories, or textile mills where mercury paints are not desired. For shutter and trim green paints, he advised the use of 5 per cent or more of *Paris green* (copper aceto arsenite). This compound has also been used in antifouling paints, where color is not an important factor. *Salicylanilide* has served well in lacquers and varnishes for electronic equipment where it is used in concentrations of 10 to 15 per cent (Leonard and Pitman, 1951). It is favored for its lack of corrosiveness, safety in handling, and satisfactory performance, and has been named in government specifications of products for treating electrical apparatus (Ezekiel, 1950).

For antiseptic and germicidal paints 8-hydroxy quinoline was reported (Troussaint, 1933) to be effective against *Escherichia coli*, *Micrococcus pyogenes* var. *aureus*, *Vibrio comma*, *Pasteurella pestis*, *Salmonella typhosa*, and other bacteria. Vallee (1934) concluded, however, that 8-hydroxy quinoline sulfate in paint up to 3 per cent was toxic to streptococci and staphylococci under certain conditions but was inactive against other bacteria. Wetchler, Lewis, and Battline (1936) tested numerous toxicants to render paints bactericidal. Their best results were obtained with a paint containing 4 per cent of *halogenated linseed oil*. This was claimed to have a bactericidal effect on *M. pyogenes* var. *aureus* and *S. typhosa* even after drying of the paint for 4 years. Valentine (1936) confirmed the work with halogenated oil insofar as *S. typhosa* was concerned but got lower antibacterial activity with *M. pyogenes* var. *aureus*. Epstein (1937 a, b, c) got excellent control of bacteria, yeasts, and molds when the organisms were in constant contact with the painted surface containing halogenated oil, but not after only contacting the surface for a short period. Practical tests in a hospital and breweries demonstrated that, while the halogenated oil paint retarded microbiological proliferation, growth of microorganisms occurred on the paint after 16 to 17 weeks, compared to 4 to 6 weeks for ordinary paints. Epstein and Snell (1941) concluded, however, that, while the halogenated oil is a step forward, further work will be required before a true antiseptic or germicidal paint is obtained.

## PRESERVATIVES FOR PHARMACEUTICALS AND COSMETICS

The preservation of pharmaceuticals and cosmetics represents a relatively complex problem owing to the varied nature of the products, possible incompatibilities, and the importance of psychic and toxic factors. De Navarre (1941) lists among the most useful preservatives the esters of p-hydroxybenzoic acid, formaldehyde, benzoic acid, and alcohol. Sokol (1952) lists alcohol, phenol or cresol, benzoic acid and sodium benzoate, salicylic acid and its derivatives, esters of p-hydroxybenzoic acid, chlorobutanol, quaternary ammonium salts, formaldehyde, sodium bisulfite, boric acid, chloroform water, mercuric cyanide, phenylmercuric salts (such as the acetate, borate, nitrate, and chloride), Merthiolate, and Metaphen.

### ETHYL ALCOHOL

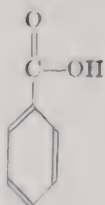
Other than sugar and salts (which are used in high concentrations to prevent microbial growth by raising the osmotic pressure of the medium) alcohol is probably the oldest pharmaceutical preservative. Gabel (1921) found that 15 per cent alcohol was sufficient for preservation against both molds and bacteria in an acid solution but in a neutral or alkaline solution containing nutrients for microorganisms a minimum of 17.5 per cent alcohol was required. A study of the composition of preparations on the market and in the compendia (Bandelin, 1950-51) confirms, from practice, the reliability of the figures given by Gabel. According to Bandelin, the quantity of alcohol may be reduced depending upon the nature of the preparation. If the preparation contains a high concentration of sugar, lesser quantities of alcohol are necessary. Sugar syrup of 65 to 68 per cent, or greater, requires no alcohol or other preservative. The greater the solids content of the preparation, the less alcohol necessary. Likewise, glycerol helps in preservation, although De Navarre (1941) states that, by itself, 50 per cent is required. Bandelin gives methods for computing the approximate quantity of alcohol to be used for preserving different preparations.

### BENZOIC ACID

Benzoic acid occurs naturally in certain foods as prunes and cranberries and has been permitted as a preservative in foods. Like other organic acids, it is active mainly as the free acid and not as the ion. For that reason it is effective mainly in acid solution, where it is essentially nonionized. In neutral solution, where the percentage



of free acid is reduced, higher concentrations are required. On the alkaline side, where it is principally in the ionic form, the compound

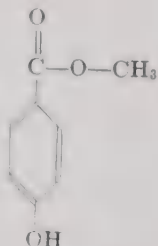


Benzoic acid

fails to preserve. In acid solution 0.1 per cent was required for preservation, in neutral solution 0.2 per cent was needed, whereas it was entirely ineffective in alkaline solution (Gabel, 1921). The same general observation applies to sodium benzoate. The effect of pH was noted earlier by Herter (1910) and confirmed later by Cruess and Richert (1929). The latter found 0.06 per cent sodium benzoate prevented fermentation by wine yeast at pH 3.0, 0.1 per cent at pH 4.5, while at pH 7.0 as much as 3 per cent permitted fermentation to proceed. In honey, 0.05 per cent sodium benzoate or 0.06 per cent sodium salicylate gave preservative action. Benzoate, however, was permissible for use at a maximum of 0.1 per cent whereas salicylate was not permitted in concentrations exceeding 0.02 per cent (Lochhead and Farrell, 1930). Benzoic acid has a further advantage over salicylic acid. Benzoic does not irritate the skin and has no keratolytic effect, whereas over 0.5 per cent salicylic acid has a definite keratolytic effect (Suess, 1936). Gershenfeld and Perlstein (1939) showed that 0.1 per cent of sodium benzoate or sodium salicylate prevented attack of a gelatin solution at pH 3 to 4, but neither preserved a gelatin solution buffered at pH 7 to 8.

#### ESTERS OF PARAHYDROXYBENZOIC ACID

The esters of p-hydroxybenzoic acid have received the greatest attention and the widest use, excepting alcohol, of any of the preservatives of the pharmaceutical and cosmetic industries. Neidig and



Methyl parahydroxybenzoate

Burrell (1944) cite 377 references to these esters as preservatives, covering a period only to 1944. It is generally agreed that their

properties, which include effectiveness against bacteria and molds in low concentration, lack of color and odor, low toxicity and absence of skin irritation, make them particularly well suited for use in pharmaceuticals and cosmetics. They are more active against microorganisms than are the corresponding esters of benzoic or salicylic acid. The activity of the esters against bacteria and fungi increases with increasing molecular weight to, at least, the butyl ester. Because these compounds are very weak acids, they are effective further into the neutral and alkaline range than stronger acids such as benzoic acid, although their greatest activity is in acid solution where they are not ionized.

The esters commonly employed are methyl, ethyl, propyl, and butyl. The water solubility decreases as the molecular weight increases. The benzyl ester is more toxic than the others, but it is so insoluble that its use is limited. The sodium salts have greater water solubility than the unreacted esters and may be used to make concentrated aqueous solutions of the preservatives. Because of its greater water solubility, the methyl ester is often used for aqueous preparations while the butyl ester is employed for oily materials. The propyl ester is popular because it is in a compromise position regarding water and oil solubility and because it has the greatest activity for the least toxicity, compared with the other esters.

Considerable data on these esters are found in the reports of Suess (1936), De Navarre (1941), Neidig and Burrell (1944), Bandelin (1950-51), and Sokol (1952). The paper by Sokol (1952) gives a great deal of original data on the antibacterial and antifungal properties of the esters and on their toxicology. Accumulated data from numerous reports given by Neidig and Burrell show that a range of 0.02 to 0.15 per cent of the methyl, ethyl, propyl, and butyl esters is required, depending on the type of preparations to be protected and the organisms involved. Suess gives recommended quantities ranging from 0.1 to 0.2 per cent. Sokol gives experimental data supporting these values. Tests on acute and chronic toxicity, allergenicity, and the metabolic disposition of the p-hydroxy-benzoic acid esters reported by Sokol implies the degree of safety in the use of these compounds. Further evidence has been implicit in their permitted use as food preservatives in Germany.

#### PHENYLMERCURIC SALTS

Because of the danger associated with the use of mercury, manufacturers have been discouraged in the use of mercurials

where other preservatives could be substituted. Nevertheless, the great bacteriostatic and fungistatic power of phenyl mercurials has stimulated their use in certain applications. Phenylmercuric borate has been used at a concentration of 0.002 per cent as a preservative for dried human blood plasma (De Navarre, 1943; Berk and Company, 1944). Considering that this product goes directly into the blood stream, the danger can be discounted when the concentration of the mercurial is sufficiently low. The phenylmercuric salts, the nitrate, borate, and picrate, have been approved for specific applications by the American Medical Association (1941). Gibbs *et al.* (1941) concluded that the constant ingestion of small amounts of mercury is without toxicological significance, for mercury, contrary to popular opinion, is not stored in the body as in the case of lead. Many foods contain small quantities of mercury and normal human excreta contain about 20 micrograms of mercury per day.

Pharmaceutical grades of the following phenylmercuric salts are commercially available: the acetate, borate, benzoate, chloride, gluconate, nitrate, and salicylate. These compounds are colorless and odorless. The phenylmercuric cation is the toxic entity, the anion serving only to effect solubility. They are active against gram-positive and gram-negative bacteria, as well as fungi, but their inhibiting power far exceeds their killing power. De Navarre (1943) stated that, when 1 part of phenylmercuric benzoate was put into 35,000 parts of a cosmetic cream, it was found that the cream became free of viable organisms in 20 to 30 minutes after having been inoculated with *M. pyogenes* var. *aureus*. Eye washes and drops, lubricating and nasal jellies, gelatin waving solutions, glue sizing and starch paste, and bentonite gels require from 0.001 to 0.004 per cent of phenylmercuric compounds for preservation. Mercurials combine with amino acids and sulfides to form insoluble, inactive derivatives and are therefore ineffective in preparations containing these groups of compounds.

## PULP AND PAPER PRESERVATIVES

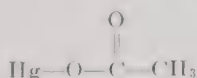
Preservatives are required in many stages of the process for manufacturing paper. Bacteria, molds, and yeasts may grow in the pulp to sour it, stain it, and reduce the quality of the paper. In the processing equipment a complex microbiological slime clogs pipes, screens, and filters. Buckman (1949) estimated that the economic loss caused by slime in a paper mill may be from 1 to 5 dollars per ton of pulp processed. Sanborn (1944, 1951) studied the slime problem and stressed the importance of cleanliness. He



(also Appling *et al.*, 1951) found that the large masses of slime must be removed before chemical agents are effective. Shema *et al.* (1949) listed the more effective chemicals as follows: (1) chlorine or chloramine, (2) mercurials, (3) chlorinated phenols, (4) quaternary ammonium compounds, and (5) various combinations of these materials.

In testing 130 compounds, Sanborn found 25 to show promise. These included halogenated phenols, their metal salts, and similar substituted derivatives. In a series of reports of laboratory tests of disinfectants for use in the pulp and paper industry, Appling and McCoy (1944, 1945 *a, b*) considered the compounds against their costs to inhibit three representative microorganisms. These were *Aerobacter aerogenes*, a gram-negative, rod-shaped bacterium commonly found in the soil; *Bacillus mycoides*, a gram-positive spore-forming, rod-shaped bacterium; and *A. niger*, a common, vigorous mold. *Ethylmercuric phosphate* and *sodium pentachlorophenate* were the most economical compounds. They were both about equal in cost to inhibit *B. mycoides* and *A. niger*, but the mercurial was only one-seventh the cost of the phenolic in the control of *A. aerogenes*. The latter organism was the most costly of the three to inhibit with most compounds. Other industrial organic mercurials such as *phenylmercuric acetate* and *pyridylmercuric acetate* were also included in the tests.

The compound and quantity to be employed may depend on the specific mill problem, for different microorganisms may give trouble in paper mills. The conditions of acidity are also important in this consideration for, as shown by Appling *et al.* (1947), sodium pentachlorophenate was most effective in the more acid solution, a quaternary ammonium compound in the more alkaline solution, whereas the organic mercurials did not appear to be affected in



Phenylmercuric acetate

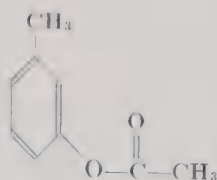
activity over a range of pH 4 to 8.5. In mill tests, the latter workers found that the alternate use of sodium pentachlorophenate with ethylmercuric phosphate clearly indicated an improvement in slime control over the use of the latter compound alone. King and Venables (1949) also reported successful control in the mill with the alternate use of different bactericides and fungicides and frequent cleanups. Holmes (1941) observed that chlorophenol salts

are not used up by organic matter in the wash water as is chlorine but tend to concentrate and remain in the system. By both chemical and biological assay, Halliday (1950) demonstrated that phenylmercuric ions are adsorbed by cellulose fibers and are not distributed evenly throughout the processing system. They reported that a water-resistant lacquer containing phenylmercuric acetate kept treated surfaces free of slime for about 3 months.

Ballman and Smith (1943) mention various additional applications for the chlorinated phenols in the pulp and paper industry. Sodium tetrachlorophenate was reported to preserve stored pulp, while sodium pentachlorophenate was found to extend the useful life of woolen felts. Starch and casein solutions used for coating paper are subject to microbial decomposition. Sodium trichlorophenate was said to be best for preservation of casein and sodium pentachlorophenate best for starch solutions. Soap wrappers, gummed tape, and insulation board are treated with phenolics such as the sodium salts of orthophenyl phenol and pentachlorophenol to prevent attack by fungi. Mold-resistant paperboard egg-crate fillers and flats keep eggs free of mold during cold storage, according to Mallmann and Michael (1940). Although sodium trichlorophenate was found superior for this purpose, sodium pentachlorophenate was used commercially because of its lesser odor, greater permanence, and lower cost.

## PRESERVATIVES FOR OPTICAL INSTRUMENTS

Fungus damage to optical equipment of all types in tropical climates is a serious problem which was dramatically emphasized during the war when large quantities of optical equipment were sent to the South Pacific area. According to reports (Waksman *et al.*, 1944), a random inspection of service binoculars by Australian workers showed more than half to be overgrown with fungi. Mold does not obtain nourishment from the glass itself, but from organic dust and grease or the leather instrument case. Fungus mycelium



Metaeresyl acetate

on the lenses obscures vision and may make it necessary for equipment to be sent back to the factory. After long periods, the organic acids produced by the fungus may etch the glass lenses and permanently damage the instrument (Fig. 21).

The problem has been attacked from various angles, but the chemical method appears to be the most satisfactory. Best control was given with *Metacresyl Acetate* (Cresatin). This is a volatile fungicide employed by mixing with an equal quantity of ethyl cellulose resin and enclosing the resultant taffy-like product in an aluminum capsule which is placed in the instrument case. One-half million of these capsules were procured by Frankford Arsenal and distributed throughout the Pacific area for application to instruments during servicing and repair (N.D.R.C., 1946). In Panama

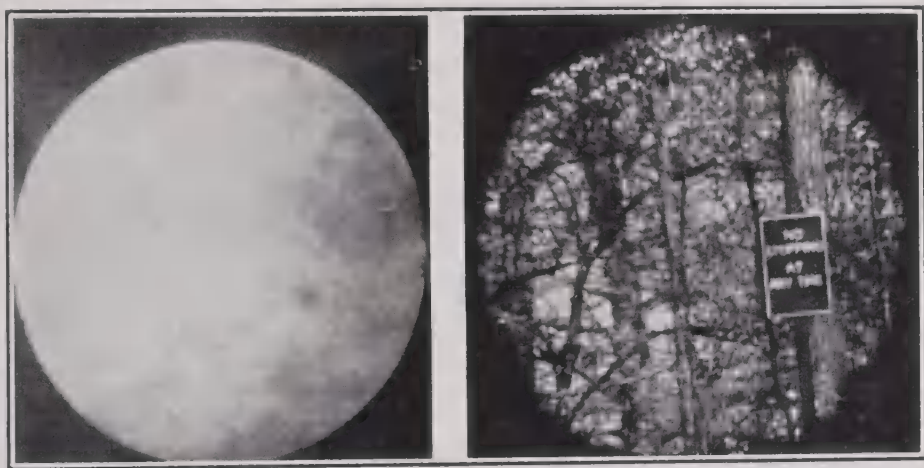


Fig. 21.—The effect of alpha radiation of radium in preventing fungus fouling of optical instruments. At left is view through an untreated telescope after two months in a tropical testing chamber. At right is view through a telescope protected with radium foil. (Courtesy U. S. Corps of Engineers).

Canal Zone exposure tests, the m-cresyl acetate capsules were the only means found to be completely effective in controlling fungus inside binocular cases (Teitell and Berk, 1952). It was noted, however, that cupric oxide blackened copper alloy prism shields were corroded by m-cresyl acetate.

Reference to Australian investigations (N.D.R.C., 1945) is made of the recommended use of sodium ethylmercuric thiosalicylate (Merthiosal or Merthiolate). This compound was incorporated to the extent of 0.2 per cent in lacquer used to paint interior surfaces of instruments and was mixed with the cement and luting compounds. The methyl and butyl derivatives have also been used with success, although the former was observed to induce corrosion of aluminum. In the Canal Zone tests of Teitell and Berk (1952), however, sodium ethylmercuric thiosalicylate-treated binoculars



became moldy in large numbers. Vicklund (1946) reported on an interesting method for prevention of the fungus fouling of optical instruments. Metal foil treated with 15 micrograms per square inch of radium sulfate and used to surround the lenses prevented fungus growth as a result of the alpha radiation. Teitell and Berk confirmed the protective properties of the radioactive foil but indicated a possible health hazard in the storage of foil-treated instruments. Coating camera lenses with a hard fluoride coating did not prevent mold growth, but use of a quaternary ammonium salt in an antifogging compound applied to the lens surfaces kept the lenses mold-free for over a year in Panama (N.D.R.C., 1946).

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## 30

# GASEOUS STERILIZATION

## INTRODUCTION

GASEOUS sterilization may be considered a rather new field in bacteriology. Certainly this is true to the extent that the term should be defined before the subject can be adequately discussed. As referred to in this chapter, gaseous sterilization is concerned with the treatment of solid objects or materials with a chemical in the gaseous or vapor state to destroy all living microorganisms with which they may have been contaminated. Thus gaseous sterilization is a specialized type of chemical sterilization, the distinction being that the chemicals used are gases, not liquids or solids in solution. In recent years new techniques have been developed bringing such procedures into prominence, primarily because they enable one to sterilize many types of objects or materials which cannot be treated by ordinary means.

Sterilizing procedures, as opposed to less drastic sanitizing or disinfecting procedures, have traditionally placed primary emphasis upon heat, either moist or dry. Autoclaving, canning, boiling in water, or placing objects in hot air ovens are common examples. Chemical methods of sterilization have been used to a lesser extent. Such chemical methods have usually utilized strong liquid disinfectants in which the material being treated is immersed or with which it is washed. Techniques were needed which did not damage materials sensitive to heat or moisture, and which did not present the problem of removing a liquid disinfectant from the object following treatment. Chemical sterilization with gaseous agents avoids many of these difficulties. With this technique, cold sterilization or at least sterilization at room temperatures can be achieved. The fact that a gas dissipates naturally once unconfined, offers a solution to the technical problem of removing the chemical following treatment without danger of recontamination. The result is certain biological products, foodstuffs, various materials such as wool and plastics,

laboratory apparatus, electrical equipment and the like which formerly presented very difficult sterilization problems can now be treated without damage.

While this technique of gaseous sterilization is a relatively new development which only recently is being put on a firm scientific footing, the concept that vapors could possibly be used to disinfect has a very old and somewhat confusing background, which deserves some mention.

## EARLY WORK

Without attempting to give a complete historical review of the background of gaseous sterilization, passing reference should at least be made to the early activities in this field which antedate by many hundreds of years the science of bacteriology itself. Certainly the burning of incense on altars in many religious rites, the frankincense and myrrh of Biblical fame, were all associated with concepts of purification. In medieval times, during plague epidemics, records indicate that letters and messages were often held in the smoke from fires before delivery, in the hope that this would prevent the spread of the disease. Spices were sought out not only for their flavor, but also because of a belief that their pungent odors somehow reduced food spoilage. Thus, along with belief in the theory that disease was caused by miasma or evil vapors, there were attempts to counteract their bad effects with other vapors. Shortly after the promulgation of the germ theory of disease and the beginning of bacteriology as a science, Lister began spraying phenol into the air of operating rooms to cut down on surgical infections, probably the first instance of the application of a procedure related to gaseous disinfection based on any scientific evidence that it could be effective. In many of these cases, however, the object in mind was the purification or disinfection of air only, and not the disinfection or sterilization of solid objects and materials with which this chapter is concerned.

This related field of air purification by chemical means has its modern counterpart in many present day devices designed to introduce various chemicals into the atmosphere to reduce its microbial population. In some cases, unfortunately, the choice of the chemical to be added to the air is based upon scant scientific evidence as to its effectiveness. In other cases, as with the effectiveness of triethylene glycol and propylene glycol vapors, of hypochlorite mists, or aerosols of substituted phenols, a very sizeable scientific literature has grown up in recent years, based primarily on the work of Robertson and his coworkers in this country and Elford, Lovelock, Pulvertaft, Twort, Trillat and other workers abroad. Some con-

trovery still exists on the question of the effectiveness of these compounds in reducing the bacterial count of air. In the main, however, the controversy is centered around the effectiveness of various devices sold to dispense such chemicals and over the epidemiological question as to whether or not reducing the bacterial count of air in certain critical places such as barracks, public schools and hospital wards has an appreciable effect in reducing the spread of airborne infective disease. It is at least certain that the dramatic results obtained in reducing the incidence of intestinal infections by the purification of drinking water supplies have not been paralleled to date in studies on the effect of air purification techniques on the spread of respiratory infections.

This field of activity is mentioned here only because some confusion has arisen between this field of air disinfection by chemical means and the subject of gaseous sterilization. It has been shown that chemicals can be added to air in amounts sufficient to reduce its bacterial count significantly without rendering it toxic or even objectionable. In no case yet known, however, can sterilization of solid materials be achieved by exposure to an atmosphere which is non-toxic to higher forms of life. It would indeed be useful if some substance could be found which could be added to air in amounts small enough to be tolerable, and which would not only sterilize the air that we breathed, all the material objects exposed to it, but presumably also the entire respiratory system of the persons breathing the air. Nothing in our present state of knowledge indicates that such a substance can be obtained.

#### TERMINAL FUMIGATION

While much of the early work, as indicated above, led to present day developments in the field of air purification rather than towards gaseous sterilization, one particular early field of activity did bear directly on the latter subject. This was the practice of so-called terminal fumigation, or the effort to disinfect, by fumigation techniques, sick rooms following their occupancy by someone suffering from a contagious disease. This practice developed in the latter decades of the nineteenth century, became for a while a standard public health practice in many countries and was not definitely abandoned until well within this century. Such activities ceased not because it was impossible to disinfect a sick room effectively, but because of evidence that in practice terminal fumigation was often poorly done, and more to the point, our major epidemics were gradually brought under control by other means.

Terminal fumigation was accomplished by sealing up the room as effectively as possible, and admitting into it gases intended to kill off any disease germs that might still be present. Sulfur dioxide

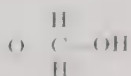
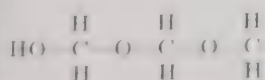


and chlorine were the main gases used at first for such practices, but their corrosiveness and damaging effect on the contents of the room certainly exercised a restraint on their use regardless of whether or not they were effective. A satisfactory agent for terminal fumigation was not found until about 1894, when within a short period of time several investigators, Rideal, Trillat, Philip, Bordet among them, showed that formaldehyde vapors could be used for such purposes. The bactericidal activity of formaldehyde had been scientifically established a few years previously, and in the next two decades hundreds of articles appeared discussing its application in sick room disinfection. An excellent summary of this early work with formaldehyde was made by Nordgren (1939), so no attempt will be made to refer to it here in detail. Much of it was concerned either with discussions of the relative merits of different methods for generating formaldehyde gas, or with the results of practical experiments, often-times crude, in which various contaminated samples were placed in closed rooms and examined after the room was treated with formaldehyde gas.

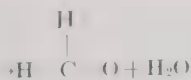
Three quite important facts concerning the action of formaldehyde as a gaseous fumigant were well established during this period, however. These were that the success of the procedure was dependent upon an elevated temperature and a high relative humidity, and that the penetration of formaldehyde into porous materials was quite uncertain.

## FORMALDEHYDE

Pure formaldehyde gas,  $\text{CH}_2\text{O}$ , is stable in high concentrations only at temperatures of  $80^\circ \text{C}$  or above. At ordinary temperatures the gas polymerizes, condensing out a white film on any available surface, so that high concentrations of the gas cannot be maintained at room temperatures. There are several types of solid polymers of formaldehyde, some with a cyclic structure as trioxane, others with a linear structure such as the polyoxymethylenes. Paraformaldehyde, the important polymer, is a mixture of polyoxymethylene glycols which has the typical structure given below, and contains 93 to 99 per cent formaldehyde, the rest being combined water. This is a colorless solid, which at ordinary temperatures gradually gives off gaseous formaldehyde, so it possesses its same sharp irritant odor. When heated it depolymerizes rapidly, giving off formaldehyde, and a little water vapor.



Paraformaldehyde



Formaldehyde gas also dissolves readily in water to the extent of about 37 per cent at room temperatures. In water solution, it

exists mainly as the hydrate  $\text{HO}-\overset{\text{H}}{\underset{\text{H}}{\text{C}}}-\text{OH}$ , with some higher hy-

drates probably also present. Commercial formalin is such a solution which contains also certain stabilizers, 8 to 15 per cent methanol being the one most widely used, to prevent the formation of solid polymers if the solution is chilled or is kept for too long a time.

Paraformaldehyde and formalin are the two commercial sources from which one obtains formaldehyde, since the compound cannot be maintained at ordinary temperatures in the pure unpolymerized state either as a gas or compressed into a liquid. These were the sources available also to early workers in this field and as mentioned previously, many ingenious methods were devised then for releasing formaldehyde from them as a gas into the area to be treated. Paraformaldehyde needs only to be heated to release formaldehyde, and various lamps in which this could be accomplished were used around the turn of the century. Paraformaldehyde was also incorporated in the wax in candles which would release the gas while burning, although of course in rather uncertain amounts since much was actually burned rather than vaporized in the process. Sulfur had also been incorporated in candles to release sulfur dioxide in the same manner. In present day practice any hot plate or electrical heating device will accomplish the same end more simply.

Formalin also releases formaldehyde gas upon heating, although when allowed to evaporate at room temperature water escapes faster than formaldehyde, and solid formaldehyde polymers are left behind. When heated, both water and formaldehyde escape rapidly and again any convenient method of boiling the solution will serve to release formaldehyde gas together with water vapor from formalin. When terminal fumigation was a common practice electrical devices for boiling formalin were not available so several other practices of dispersing formalin were followed. Spraying it directly into a room was not too effective, for unless the spraying device produces a very fine aerosol, many droplets fall before evaporation, and these evaporating naturally will leave a solid deposit of polymerized formaldehyde behind. Sheets were often wet with formalin and hung up to dry, but again not all the formaldehyde escaped into the air, since evaporation was still at room temperature. The one procedure which gained most widespread application because of its simplicity was to pour formalin over chemicals which either by oxidation or hydration produced heat and helped evaporate the liquid. The most widely used compound for this

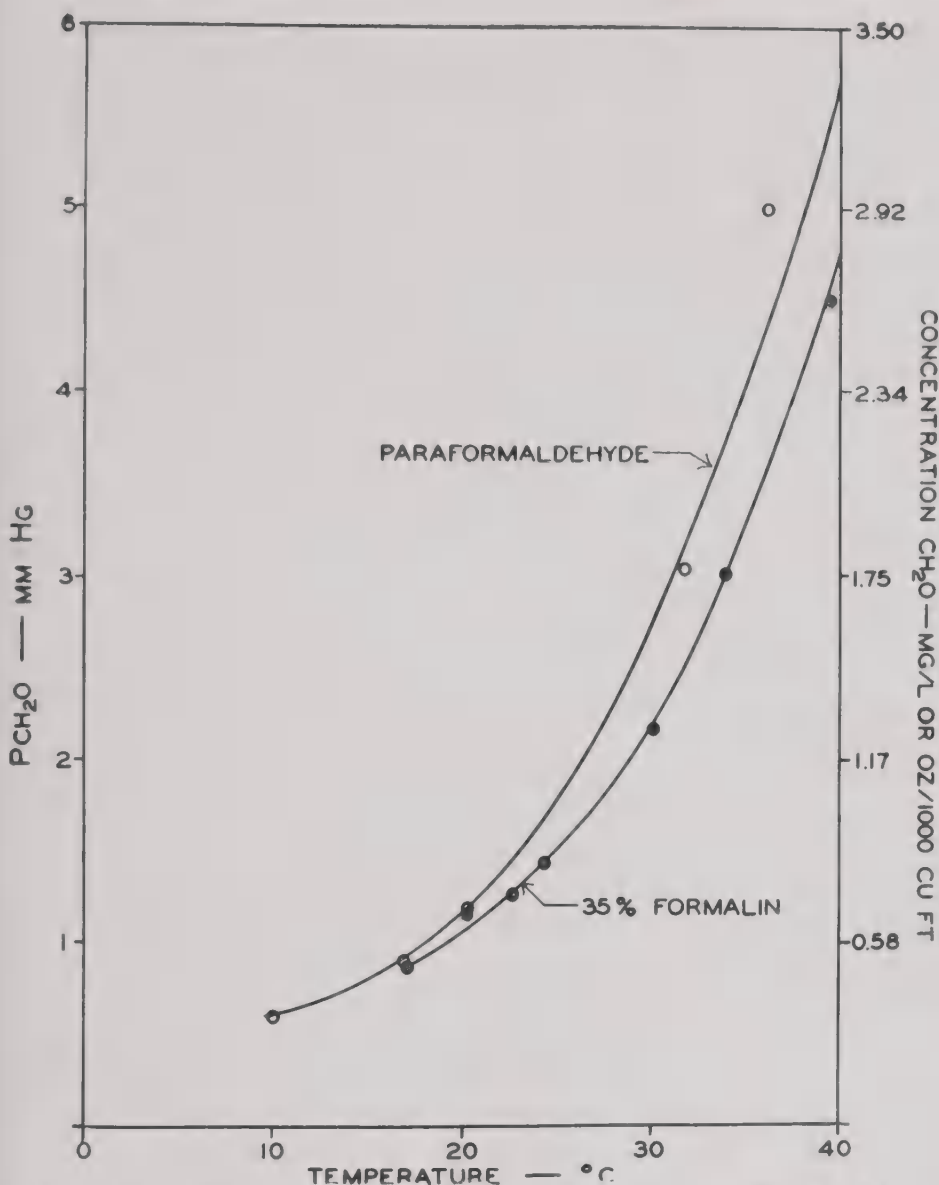


Fig. 22.—Concentration of formaldehyde gas in equilibrium with paraformaldehyde or formalin at various temperatures.

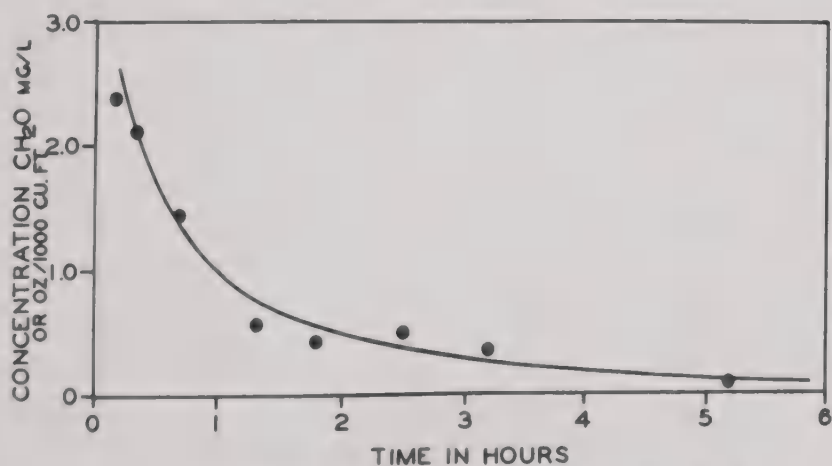


Fig. 23.—Drop in concentration of formaldehyde vapor in air in an enclosed space with time.



purpose was potassium permanganate in crystal form, using roughly twice as much formalin by weight as permanganate. Part of the formaldehyde in the formalin is oxidized by the potassium permanganate and enough heat is formed in the process to evaporate the remainder. It is difficult to calculate how much formaldehyde gas is released and how much is destroyed in such a process, but by trial and error procedures were worked out which were satisfactory.

Formaldehyde can be generated directly by the catalytic oxidation of methyl alcohol and special lamps were at one time used where methyl alcohol vapors were heated and mixed with air passed over a platinum coil. Certain articles claimed special properties for the "nascent" formaldehyde thus formed, but the procedure was inefficient and never widely used.

In only a few of the instances reported in early literature was any attempt made to determine just what amount of formaldehyde was actually introduced, and just how much of this stayed in the gaseous state.

The vapor pressure of formaldehyde gas either over paraformaldehyde or over 37 per cent formalin is about the same, and appears in graphic form (Fig. 22) from the data of Nordgren (1939).

The concentrations in this graph\* represent the maximum amounts of formaldehyde which can be maintained in stable form in air at the indicated temperatures. Thus at room temperature only about 3 mg of  $\text{CH}_2\text{O}$  vapor can be maintained at equilibrium per L of space. Any concentration above that is unstable and will leave the atmosphere, either by polymerization into a solid, or by dissolving into any moisture present. In actual practice, as shown in figure 22, not even this amount in air can be readily maintained unless a large excess of paraformaldehyde or formalin is present. In figure 23 appear unpublished data from this laboratory which show the typical change in concentration of formaldehyde in air when formalin is vaporized into a small room in amounts which correspond to an initial concentration of about 7 mg/liter. Air samples taken a few minutes following its introduction show the presence of only 2.5 mg/liter and this concentration, although below equilibrium concentration, drops steadily over a matter of hours. Similar calculations show that the amount of water vaporized into

\*Concentrations are given in this graph as both milligrams per liter and ounces per 1000 cubic feet. It is one of the few happy coincidences of the English and metric systems of measurement that these units are for all practical purposes identical, differing by less than 1 per cent. Because of this coincidence, gas concentrations will be expressed in these units throughout this chapter. Air itself at one atmosphere pressure and room temperature weighs roughly 1200 mg per L, or ounces per 1000 cubic feet. The concentrations given for these gases in units of weight per volume of space can be converted to percent in air values if desired, using this figure.

the room with the formalin exceeded the dew point so that the total amount of water vapor held in the air was also less than that introduced. Since neither the formaldehyde nor the water escaped from the room at rates which would account for this rapid disappearance from its atmosphere, and since both were introduced in amounts over their equilibrium concentration in air, the moisture and the formaldehyde both must have condensed out on the various surfaces within the room. Thus, gaseous sterilization with formaldehyde is somewhat of a misnomer, since introduction of formaldehyde gas into a closed space serves mainly as a mechanism for distributing either solid formaldehyde polymers or moisture films in which formaldehyde is dissolved over all available surfaces within the enclosed space, depending upon whether the gas was introduced dry or with excess water vapor.

These particular observations are not new. They were pointed out several times in the older literature and served to explain why high moisture content was necessary for rapid sterilization, since this resulted in effect in treating the surfaces with an aqueous solution of formaldehyde rather than with solid paraformaldehyde, and why penetration into and complete sterilization of porous substances was usually poor, since most of the formaldehyde remains on the most readily available outer surfaces where it first condensed from the air.

Although formaldehyde is not used to the extent that it was previously, when terminal fumigation of sick rooms was a common practice, it still finds some use today. The sterilization of rooms or other large enclosed areas is still accomplished most readily with this technique although it is done more frequently in laboratories or industrial areas where surface contaminants must be kept to a minimum, than in hospital rooms. Special uses are the sterilization of certain types of surgical or medical equipment in variously designed small cabinets (Goedrich and Schmidl, 1942), and the treatment of raw wool in this manner to prevent anthrax infections among wool sorters. Salle and Korzenovsky (1942) point out, as several other authors have done, how penetration can be improved by utilizing a vacuum chamber.

Precise data on the bactericidal activity of gaseous formaldehyde are difficult to obtain, because the concentration of the gas as measured in the atmosphere is not meaningful, since most of the formaldehyde introduced quickly leaves the atmosphere and is adsorbed on exposed surfaces, where its concentration cannot readily be determined. Nordgren (1939) reviewed earlier attempts to make such measurements. In his own experiments suspensions of bacterial spores were placed on glass slides and allowed to dry for

15 to 20 minutes. He states that the test objects were dry under these conditions but the suspensions themselves were "only slightly dried up." Such slides were placed in flowing atmospheres completely saturated with water vapor and containing measured concentrations of formaldehyde vapor. Such conditions were ideal for the rapid pickup of formaldehyde and extra moisture by the organisms from the atmosphere, and in many of his experiments spores were killed in between 20 and 45 minutes at temperatures around 20° C, and with partial pressures of formaldehyde ranging between 0.6 and 0.8 mm Hg or concentrations just over 1 mg/liter or 1 oz/1000 cubic feet. In actual practice where formaldehyde is introduced into a closed space considerably longer exposure times are usually required for sterilization of exposed surfaces. Under such conditions the amounts available for becoming adsorbed on surfaces are limited, and even though the concentration of the gas in the atmosphere may be higher than 1 mg/liter for some time as indicated in figure 23, spores may survive for several hours.

Numerous authors on the other hand have reported on the bactericidal activity of aqueous formaldehyde solutions. Tilley (1945) reported coefficients of dilution as near unity and temperature coefficients ranging from 2.8 to 3.7 for such solutions with several vegetative organisms. He mentions that it is not because of a steep temperature coefficient that sterilization with gaseous formaldehyde must be kept above 18° C but rather because of the chemical properties of the gas and its tendency towards increased polymerization at the lower temperatures. Phillips (1952) reported briefly that bacterial spores were only some 2 to 15 times as resistant towards formaldehyde as were vegetative bacteria, a ratio much lower than is the case with many other types of disinfectants.

Experiments in this laboratory have shown that sterilization of enclosed spaces may be most readily accomplished by vaporizing liquid formalin into the space at a rate of  $\frac{1}{2}$  to 1 ml for each cubic foot of enclosed space. This gives a theoretical dosage of 7 to 15 mg/liter of formaldehyde. Where the relative humidity of the air is below 70 per cent, additional moisture is also vaporized into the space. The temperature of the space is maintained at least at 20° C, preferably higher. Within an hour or two vegetative bacteria will be destroyed, but the space must be kept closed for as much as 12 hours if a high concentration of bacterial spores is present.

Using this technique complete sterilization of all well exposed surfaces is obtained, but it is difficult to sterilize completely surfaces covered in any manner or to sterilize throughout porous materials. One advantage of this technique is that the space need only to be relatively tightly enclosed, not necessarily hermetically sealed, a



condition often impossible to achieve when entire rooms are to be treated.

One of the advantages mentioned in the introduction for gaseous sterilization agents in general is lacking with formaldehyde. It is often quite difficult to remove the compound following treatment. Prolonged airing, up to several days, is often required, unless this process is speeded up by heat, or efforts are made to neutralize residual formaldehyde. Ammonia gas has been used for this purpose, since it combines with formaldehyde to form hexamethylenetetramine but this is not always completely successful. The reason for this persistence of formaldehyde following treatment lies in the fact that it does not remain in gaseous form during sterilization but becomes adsorbed on exposed surfaces as previously explained. What gas remains in the atmosphere following treatment quickly dissipates. It is the adsorbed film of polymerized formaldehyde which takes so long to remove.

## ETHYLENE OXIDE

The use of ethylene oxide vapor for gaseous sterilization is a considerably more recent development than is the case with formaldehyde. Most interest in the use of this compound has been in comparatively recent years, although Phillips and Kaye (1949) in reviewing the literature on the bactericidal activity of ethylene oxide found references back as far as 1929. Occasional articles appeared in the literature from that time on, and quite a few patents were taken out on sterilizing procedures utilizing ethylene oxide, particularly the series by Griffith and Hall (1940 *a, b, c*, 1943).

Ethylene oxide is a colorless gas at ordinary temperatures, liquifying readily at  $10.8^{\circ}\text{C}$  and freezing at  $-111.3^{\circ}\text{C}$ . The liquid is miscible with water in all proportions as well as with all common organic solvents. The compound has a rather pleasant ethereal odor, and an inhalation toxicity about that of ammonia gas. It can also act as a skin vesicant, particularly if aqueous solutions are spilled on clothing (Sexton and Henson, 1949, 1950) or materials in which it is adsorbed are held against the skin (Phillips and Kaye, 1949). It has the formula  $\text{CH}_2 - \text{CH}_2$ , and as such represents



the simplest cyclic ether or epoxy compound. The compound, like formaldehyde, is manufactured on a very large scale, largely for use in synthesis of other organic chemicals, and is available both in the pure state compressed to a liquid in cylinders, or as a mixture containing 10 per cent ethylene oxide and 90 per cent carbon dioxide

under the trade name Carboxide.\* The reason for the availability of the latter mixture lies in the fact that ethylene oxide is vigorously flammable and as little as 3 per cent of its vapors in air will support combustion, with explosive violence if confined, behaving in this respect much like ordinary diethyl ether. This can be prevented if sufficient carbon dioxide is present, and a 10 per cent mixture of ethylene oxide in carbon dioxide can be mixed with air in all proportions without ever passing through a flammable range (Jones and Kennedy, 1930). For this reason pure ethylene oxide gas is used as a sterilizing agent only in small cabinets where a relatively few grams of the compound are involved (30 grams is the top limit of the pure compound allowed in these laboratories), or in vacuum chambers where air is removed before the pure gas is admitted. In larger chambers with air present Carboxide is indicated as a safety precaution. The action of the vapor is slow, as the data in the following table show:

TABLE 107.—TIME REQUIRED FOR THE STERILIZATION OF *Bacillus globigii* SPORES ON COTTON CLOTH WITH ETHYLENE OXIDE GAS

Conc. of gas mg per L or oz per 1000 cu ft	Exposure time (hours)							
	$\frac{1}{2}$	1	2	4	6	8	10	24
Temperature: 37° C								
22.1			+++	++	+	0	0	0
44.2			++	+	0	0	0	0
88.4		+++	+	+	0	0	0	0
442	+	0	0	0	0			0
884	0	0	0	0	0			0
Room temperature: 25° C								
22.1			+++	+++	+++	+++	+++	+
44.2			+++	+++	++	++	+	0
88.4			++	++	+	+	0	0
442	+++	++	+	0	0			0
884	+++	++	0	0	0			0
Temperature: 5° C								
	24 hours	48 hours	72 hours					
22.1	+++	+++	+++					
44.2	+++	+++	+++					
88.4	+++	++	+					
442	0	0	0					
884	0	0	0					
+++ Less than 99 per cent reduction (data of Phillips, 1949)								
++ Reduction between 99 and 99.9 per cent								
+ Reduction greater than 99.9 per cent								
0 No organisms recovered								

Unlike formaldehyde, ethylene oxide gas is more reactive at lower relative humidities (Kaye and Phillips, 1949), at least over the range of 28 to 97 per cent relative humidity. There is some

\*Carbide and Carbon Chemicals Corporation, New York, New York.

indication that at extremely low relative humidity, or under conditions of extreme desiccation such as one encounters following lyophilization, this action is reversed and the bactericidal action of the compound is lessened.

The advantages of ethylene oxide sterilization lie not in the speed, simplicity or inexpensiveness of the treatment but rather in the fact that many types of materials are sterilized with least damage to the material itself using this technique. Among such materials are spices and other foodstuffs (Yesair and Williams 1942; Hall, 1951), unbroken eggs (Lorenz, Starr and Bouthilet, 1950), hospital bedding and books (Kaye, 1950), plaster bandages (Wilson, 1950), penicillin (Kaye, Irminger, and Phillips, 1952), other types of medical or biological preparations, (Griffith and Hall, 1949 *b, c*), culture media (Wilson and Bruno, 1950; Hansen and Snyder, 1947), soil (Clark, 1950; Allison, 1951), plastics (Engley, 1951), and laboratory equipment both mechanical and electronic (unpublished data, Phillips).

The penetrating ability of this gas is remarkable, being quite unlike formaldehyde in this respect. Phillips (1950) gave data published in abstract form only showing that cotton cloth impregnated with bacterial spores and placed inside unsealed paper envelopes could be sterilized by exposure to ethylene oxide vapor at about 450 mg/liter concentration and room temperature in from 4 to 6 hours exposure time, even though the envelopes were set between many layers of clothing. Lorenz *et al.* (1950), reported that the gas penetrated through unbroken egg shells, sterilizing the contents within. With vacuum treatment penetration is achieved through whole barrels of spice (Personal communication; McCormick and Co.).

There are limits to this ability to diffuse and penetrate, however. Phillips (1950) reported a simple experiment in which 5 ml of an identical suspension of *B. globigii* spores in distilled water were placed in a petri dish and in a test tube respectively and exposed simultaneously to ethylene oxide vapor. At the end of 4 hours the suspension in the petri dish was sterile while the suspension in the test tube was reduced only about 50 per cent in count, indicating that the gas diffused through the shallow layer of water in the petri dish, but only about halfway down through the liquid in the test tube. This ability of the gas to diffuse and penetrate so well through various types of barriers also presents a problem in its practical application, since exposure cabinets or chambers must be very tightly sealed if the concentration is to be maintained for the several hours usually required for treatment. It is usually impossible to make an ordinary room tight enough to retain the gas,



and for this reason, as well as because of cost factors and flammability hazards, formaldehyde rather than ethylene oxide is used in fumigation of large interior spaces.

This same ability of ethylene oxide to diffuse and penetrate rapidly results in a very quick loss of any residual ethylene oxide once the objects being treated are removed from its atmosphere. It is quite unlike formaldehyde in this respect. Many solid objects can be wrapped in paper and tied loosely before treatment, much as objects are prepared for autoclaving. Penetration of the gas through the wrapping presents no problem and when removed from treatment the residual gas quickly dissipates. The paper covering then allows the object to be handled without becoming recontaminated. Exceptions to the quick loss of residual ethylene oxide following treatment, have been found with rubber and certain plastics. Ethylene oxide is soluble to a certain extent in these organic solids and quite some time is required for the gas to diffuse back to their surfaces and out to the atmosphere. The author has found as much as 4 per cent ethylene oxide present in rubber immediately following sterilization, measurable amounts still remaining after 5 hours airing, but none present 24 hours following treatment. Laboratory personnel have received chemical burns by donning rubber shoes only an hour or so after they have been sterilized. Presumably, certain organic liquids or oils could similarly retain ethylene oxide for appreciable lengths of time.

Many workers have reported directly or by implication on the fact that sterilization is not hampered greatly by the presence of large amounts of extraneous materials present during treatment. The success in sterilizing eggs, biological media and soil already referred to are indications of this fact. Phillips (1950) mentioned that dry bacterial spores mixed with such materials as raw eggs, feces, vasoline, and motor oil could be sterilized by exposing the mixture to an atmosphere containing ethylene oxide, provided the depth through which the gas had to diffuse was not too great. Trouble has been reported, however, in the sterilization of certain foodstuffs with a high fat content. Here the difficulty appears to have been a change in the foodstuff possibly due to a reaction between the ethylene oxide and the fat, rather than difficulty in achieving sterility.

Ethylene oxide is apparently effective against all types of microorganisms. The older literature containing references to its effect on many types of bacteria and molds is covered in the review of Phillips and Kaye (1949). Since then Ginsberg and Wilson (1950) have reported on the activity of ethylene oxide solutions against several viral agents and Mathews and Hofstadt (in press

1953) on the effect of the gas on 15 animal viruses as well as several bacteria and fungi. Wilson and Bruno (1950) mentioned that *Mycobacterium tuberculosis* could be sterilized, and Kaye (1950) reported the same fact for the BCG strain. Phillips (1952) stated that bacterial spores are only several fold more resistant to ethylene oxide than are vegetative bacteria, whereas, with chlorine, the phenols, and other common types of disinfectants, spores are several thousand times more resistant. All indication to date is that the effect of ethylene oxide is an irreversible one, bactericidal rather than bacteriostatic in nature.

As mentioned earlier, the ease with which this gas diffuses and the length of time which it needs for sterilization requires that it be used in very tightly enclosed spaces. Autoclaves adapted for the admission of Carboxide as well as steam are now commercially available. Kaye, Surkiewicz and Jacobson reported before the American Chemical Society in 1952 on experiments as yet unpublished in detail concerning various types of pressurized and non-pressurized fumigation chambers adaptable for ethylene oxide sterilization, and also on the use of gas-tight bags and tarpaulins to maintain the concentration of the gas around the materials being sterilized.

## OTHER GASES

Only formaldehyde and ethylene oxide are used to any great extent today for gaseous sterilization, but many other gases have been reported in the literature to possess bactericidal properties. The old-time use of sulfur dioxide and chlorine has already been mentioned, together with reference to their corrosive action which limited their application.

Ozone,  $O_3$ , has often been mentioned as a bactericidal agent, and it has found some application in preventing mold formation in such places as food storage rooms which are apt to be damp. Elford and Van den Ende (1942) reported that while ozone killed off air-borne organisms, it had little effect upon organisms on surfaces or protected by organic matter. Ingram and Haines (1949) reported upon the inhibition of bacteria in broth or on agar surfaces when exposed to ozone vapor. The former authors used very low concentrations, around 0.04 ppm or less than .0001 mg/liter, attempting to see if concentrations tolerable to man would exhibit a bactericidal effect. Ingram and Haines reported that concentrations up to 4000 ppm or up to about 5 mg/liter are necessary to inhibit growth of established broth cultures in certain cases. In any case high concentrations of ozone in air are difficult to generate, and the compound once formed is both a very strong oxidizing

agent and quite unstable. To date the main applications of ozone have been in the field of water purification.

*Methyl bromide* gas,  $\text{CH}_3\text{Br}$ , was shown by Kolb and Schneiter (1949) to be bactericidal for anthrax spores and recommended by them for the sterilization of imported wool and hair in particular. Trickel (1952) reported that it was effective against fungi. Unpublished data obtained by D. L. Jones in this laboratory confirm these findings, and the activity of the compound was measured as about 1/10 that of ethylene oxide. Methyl bromide has the decided advantage of nonflammability, however, and has about the same order of activity as Carboxide, the diluted form of ethylene oxide which must be used in many applications where flammability is a hazard. Whether or not it is as non-corrosive and nondamaging as ethylene oxide is not fully known as yet, due to limited application.

*Cloropicrin* gas,  $\text{Cl}_3\text{CNO}_2$ , has been mentioned by several authors as having bactericidal properties, and it has found some application as a soil fumigant, where, even if complete sterilization of the soil is not achieved, many phytopathological fungi are destroyed.

*Propylene oxide*,  $\text{CH}_3\text{—CH—CH}_2$ , has been mentioned by



several authors as bactericidal. Phillips (1949) and Kaye (1949) reported briefly on several compounds related structurally to ethylene oxide, and compared their activities with that of ethylene oxide. Propylene oxide was found to be less active as well as less volatile than ethylene oxide and appears to have no advantage over that compound.

*Epichlorohydrin*,  $\text{ClCH}_2\text{—CH—CH}_2$ , and *epibromohydrin* were



found to be somewhat more active in the same studies but have not been studied to any great extent.

*Ethylenimine*,  $\text{CH}_2\text{—CH}_2$ , was reported in these screening



trials to be many fold more active than ethylene oxide, and Mayo, Moser and Kaye (1952) reported to the Society of American Bacteriologists on extensive data, as yet unpublished in detail, showing that under certain conditions (*i.e.*, high relative humidity) it had more than a hundred times the activity of ethylene oxide. It, like formaldehyde, is markedly dependent upon high relative humidity, however, and moreover it is both flammable and corrosive, parti-



cularly to many metals. Its high order of activity may perhaps permit it to be used at concentrations low enough so that these later factors are not particularly objectionable, but to date it has been put to little or no practical application.

The ideal gaseous sterilizing agent is yet to be found, and in all probability will never be found. The field of gaseous sterilization is an expanding one, however, and new techniques and new compounds used for this purpose are to be expected.

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## 31

# BACTERIOLOGICAL AND SURGICAL STERILIZATION BY HEAT

### INTRODUCTION

OF THE various sterilizing agents at our disposal that of heat, particularly moist heat in the form of saturated steam under pressure, is considered to be the most reliable and the most easily controlled medium. Unlike the chemical bactericides, and other gaseous sterilizing agents, heat leaves no toxic residue. It is readily available and the economy of operation places it in a class by itself—the cheapest of all known sterilizing agents. The principal disadvantage attendant with the use of heat is that it is not well suited for the sterilization of heat and moisture sensitive materials and supplies.

Although the mechanism of the thermal destruction of bacterial life is not clearly understood, it is held by some investigators (Isaacs, 1935; Virtanen, 1934) that death is associated with the heat inactivation of certain vital enzymes in the organism. That other mechanisms may be operative when bacteria are destroyed by high temperature was advanced by Chick (1910). It is now generally accepted that the mode of action of heat on bacteria closely parallels the heat coagulation of proteins. Many workers who have investigated this phenomenon have concluded that death is caused by the heat denaturation of the proteins which make up the bacterial cell. This analogy is supported by the sudden increase in the death rate after the critical temperature has been reached; by the increase in death rate as the result of changes in the hydrogen-ion concentration of the medium; and by the fact that bacteria show a much greater resistance to dry than to moist heat.

The order of death by heat in a bacterial population is logarithmic. This means that when a suspension of bacteria is exposed to



a sterilizing influence the rate at which the individual organisms die is governed by definite laws. It follows that if the logarithms of the numbers of surviving cells are plotted against the units of time the curve obtained will be a straight line (Fig. 24). This condition descriptive of the rate of bacterial death is characteristic of what is termed a monomolecular reaction and state, *i.e.*, a reaction in which only one substance undergoes some change. In this

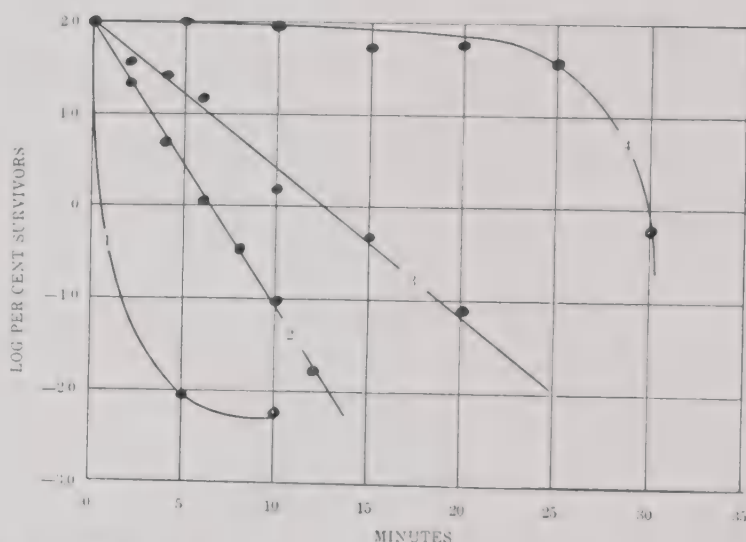


Fig. 24.—Typical death-rate curves. 1, Ascospores of penicillium at 81° C. The majority are highly susceptible with a few resistant ascospores present. 2, Putrefactive anaerobe at 115° C—an essentially uniform population. 3, *Escherichia coli* at 51.7° C—an essentially uniform population. 4, Sclerotia of penicillium at 90.5° C. (From Williams, Cameron and Williams, 1941. Courtesy of Food Research.)

connection, Rahn (1945) has stated that “the customary explanation that death is brought about by heat inactivation of the enzymes cannot be correct because, for mathematical reasons, a logarithmic order is possible only when death is due to the destruction of a single molecule in the cell.” Rahn and Schroeder (1941) have further pointed out that “it is highly improbable that this one molecule is one of the many equal enzyme molecules in the cell. It is far more probable that it is a very rare molecule, *e.g.*, a gene or an equally important molecule of the cell division mechanism.” The cause of death in sterilization by moist heat is quite different from that encountered in dry heat processes, and the rules governing the former do not apply to the latter. In brief, death by moist heat is the result of coagulation of some protein in the cell; death by dry heat is primarily an oxidation process.

## THERMAL DEATH POINT AND THERMAL DEATH TIME

The killing of bacteria by heat is a function of the time-temperature relationship employed. However, use of the term "thermal death point" (the lowest temperature at which an aqueous suspension of bacteria is killed in 10 minutes) is misleading because it implies that a certain temperature is immediately lethal to a bacterial population, without regard to the exposure period, the environment surrounding the organisms or their physiological state. When a population of viable organisms is exposed to a lethal degree of heat, the population decreases in a fairly orderly manner. Thus,

TABLE 108.—TIME-TEMPERATURE RELATIONSHIP FOR MOIST HEAT STERILIZATION  
(Compiled by McCulloch, 1945)

<i>Authority</i>	<i>° Centigrade</i>	<i>° Fahrenheit</i>	<i>Time, minutes</i>
Jordan	120.0	248	5
Muir & Ritchie	120.0	248	7½
Gerard	115.5	240	10
Eyre	115.0	239	15
Beeson	115.0	239	20
Sternberg	115.0	239	25
Novy	110.0	230	15
McFarland	110.0	230	15

if 90 per cent of a viable population is killed during the first minute of exposure, approximately 90 per cent of the survivors will be killed during each subsequent minute, which will continue until nearly all of the population is extinct. Then an exception frequently occurs in that a very few members of the population may survive longer than the above calculation would indicate.

Because thermal death time-temperature values are dependent upon a variety of factors it is difficult to obtain consistent determinations. Comparable results can only be expected when conditions are standardized as to age of culture, approximate number of cells or spores, pH of suspension, dimensions of test tubes and thickness of glass in test tubes. Whereas the thermal death point determination was originally adopted as the standard of comparison of heat tolerance of different species, this has in recent years given way to a more practical measurement designated as "thermal death time." This refers to the shortest period of time necessary to kill all bacteria in a given suspension at a given temperature.

The medium in which the organisms are suspended has an important influence on the amount of heat required to kill. With few exceptions, toxic substances become increasingly germicidal with slight increases in temperature. The effect is much greater

in the case of acids than with alkalies. It is entirely possible for some substance, which is present in such small amounts as to be stimulatory at ordinary temperatures, to be lethal at temperatures several degrees below the actual thermal death point of the organism. Products of metabolism are considerably more toxic at higher temperatures.

That authorities are not in agreement concerning the thermal death requirements of microbial life is evident from the data given in table 108.

### DEATH RATES

The rate at which a given culture will die is accelerated with each increase in temperature. Thus, the "thermal death point" of *Salmonella typhosa* is commonly given as 55° C. Actually, 120 minutes at 47° C, 48 minutes at 49° C, 18 minutes at 51° C, 7 minutes at 53° C, 2.5 minutes at 55° C, or 21 seconds at 59° C would be a more precise description of the lethal time-temperature relationship. This would indicate an acceleration of the velocity of killing of about 1.63 times for an increase of 1° C. If a culture of *S. typhosa* is heated to 55° C and subcultures taken at various times while the temperature is being raised, it is entirely probable that all subcultures made until 55° C had been reached would be viable while the culture would appear to become suddenly nonviable when 55° C had been reached. In order to avoid this erroneous conclusion, bacteriologists are in fair agreement that a thermal death temperature should be based upon the survival of a 24-hour culture of the organisms in broth, pH 7, for 10 minutes.

Before laboratory data are used as the basis for recommended sterilizing procedures, the death rate should be calculated and then sufficient additional exposure included to compensate for possible inaccuracies in the experimental observations, for the presence of abnormally resistant organisms, for the probable errors in the thermometers and other equipment used in the final procedure, and for a reasonable degree of laxity on the part of the operator.

Since sterilization by moist heat apparently follows the first order reaction law, it is customary to compute the death rate constant  $k$ . This may be represented by:

$$k = \frac{2.303}{t} \log \frac{\text{initial bacteria}}{\text{bacteria at time } t}$$

where  $t$  is the time of contact with the sterilizing agent. Higuchi and Busse (1950) have derived and verified experimentally a modified form of the classical Arrhenius equation to relate the steriliza-



tion time to the absolute temperature of sterilization. This relationship as recently discussed by Pfeifer and Vojnovich (1952) is given as:

$$\log t_d = \frac{0.219E}{T} + K,$$

where  $t_d$  is the necessary sterilization time,  $E$  is the heat of activation characteristic of killing the most thermally resistant species present, usually in the range of 50 to 100 kilocalories for the most resistant micro-organisms and spores,  $T$  is the absolute temperature of sterilization, and  $K$  is a constant depending on the number and kind of the most thermally resistant species present. A plot of the logarithm of the necessary sterilization time against the reciprocal of the absolute temperature of sterilization will give a straight line. Such data are highly useful in comparing sterilization rates and for deciding whether a higher or lower sterilization temperature is to be preferred.

## THERMAL RESISTANCE OF SPORES

Bacterial spores are recognized as the most resistant of all living organisms in their capacity to withstand destructive agents. Spores are a normal stage in the life-cycle of both the bacilli and the clostridia, and represent a phase of bacterial life in which the processes of the living cell are carried on at a minimum rate. While formerly it was believed that sporulation was stimulated by adverse conditions, such as extreme temperatures, aridity, or the accumulations of the toxic products of metabolism, it now appears that many species of bacteria sporulate in a favorable environment.

Resistance appears to vary widely from species to species and to a considerable extent within a species and within a given spore population. According to Williams and Harper (1951), the luxuriance of growth and the luxuriance of sporulation are not governing factors in determining heat resistance of spores. As is the case with vegetative cells, incubation at the optimum temperature favors increased thermal resistance. For most spores, a definite cycle of resistance appears to occur and they attain their maximum resistance several days after entering into the spore stage. Other spores, such as *Clostridium botulinum* and *Clostridium tetani*, appear to be more resistant when young.

Curran (1952) has reviewed the status of existing knowledge on this subject and the following factors are cited as significant in seeking an explanation of the mechanism of resistance:

(a) "The nature of the nutrients in the spore-producing medium is significant. With *Bacillus subtilis*, vegetable infusions and isoelectric gelatin yield spores of high thermal resistance. Media deficient in certain metallic ions yield spores of low thermal resistance—those which seem to be of particular significance are the ions of phosphate, magnesium, calcium and iron."

(b) "The greater the concentration of spores in a medium, the greater is their resistance, *i.e.*, the more heat or the longer is the time required to effect sterilization."

(c) "A major factor affecting heat resistance is the reaction of the medium. The pH of maximum heat tolerance of different species may vary from 5.0 to 8.0. Salt in low concentrations increases heat resistance for mesophilic species, both aerobic and anaerobic, but beyond 8 per cent the heat tolerance is diminished."

(d) "In general, the heat survival of spores in both nutritional and nonnutritional substrates is a function of the concentration in the 15 to 50 per cent range. Heat resistance is markedly increased when spores are suspended in oily materials and the resistance of dry spores suspended in completely anhydrous fat approaches that of dry sterilization."

The thermal resistance of spores is generally attributed to a low water content. However, certain investigators hold that most of the water present in spores is bound and, therefore, less reactive and more resistant to physical agents. For example, Henry and Friedman (1937) showed that spores of the *Bacillus* group have a bound water content of the order of 60 to 70 per cent, as compared with 3 to 21 per cent in the vegetative cell. A correlation has also been found between the specific gravity of spores and their resistance to heat, the spores with the greater density being the more resistant. The character or toughness of the exine is another factor bearing some relationship to spore resistance. According to Knaysi (1944), species which germinate by shedding their exine are more resistant than those in which the exine is wholly or partially absorbed.

The separation of sporulating organisms from non-sporulating contaminants by heating the culture to 80° C (176° F) and holding for 10 minutes is common practice. The belief that most spores survive this time and temperature is not necessarily the case, as has been demonstrated by Hodge and Knaysi (1937). The result of such a practice is the selection of the more resistant spores. (Table 109).

## HEAT INACTIVATION OF VIRUSES

The lack of quantitative studies on the thermal destruction of viruses makes any general statement regarding their resistance in-

TABLE 109.—RESISTANCE TO MOIST HEAT OF THE ENDOSPORES OF CERTAIN MEMBERS OF THE GENUS *BACILLUS*  
(After Hodge and Knaysi, 1937)

<i>Organism and age</i>	<i>Temperature ° C</i>	<i>Time of exposure, minutes</i>	<i>No. of viable endospores per ml</i>	<i>Percentage of survivors</i>
<i>Bacillus cereus</i>				
2 days at 37° C	80	0	45,000	
	80	5	4	0.009
5 days at 30° C	80	0	160,000	
	80	5	21	0.013
8 days at 20° C	80	0	360,000	
	80	5	1,580	0.43
5 days at 30° C	75	0	1,100,000	
	75	10	340	0.03
8 days at 20° C	75	0	260,000	
	75	10	3,000	1.16
<i>Bacillus megatherium</i>				
2 days at 37° C	80	0	62,000	
	80	10	110	0.18
	80	10	260	0.42
	80	0	1,500	
	80	10	1 per ml	0
	80	10	1 per ml	0
3 days at 30° C	80	0	1,020,000	
	80	10	28	0.003
	80	10	46	0.005
	80	0	250,000	
	80	10	36	0.014
	80	10	70	0.028
<i>Bacillus subtilis</i> (Marburg strain)				
2 days at 37° C	80	0	90,000	
	80	15	15,700	17.4
	80	30	13,000	14.4
	80	0	1,370,000	
	80	15	148,000	10.8
	85	0	1,370,000	
	85	10	103,000	7.5
	85	10	80,000	5.8
4 days at 30° C	85	0	975,000	
	85	10	83,000	8.5
	85	10	67,500	6.9
8 days at 20° C	85	0	101,000	
	85	10	350	0.34
	85	10	400	0.39

accurate. Whereas all appear to be inactivated by high temperatures, it is also evident that wide variations exist between different ultraviolet viruses. Rivers (1928) stated that the temperature necessary to accomplish inactivation varies from 45° to 80° C, depending upon the virus. Flexner and Lewis (1909) reported the poliomye-



litis virus to be inactivated at 50° C for 30 minutes, but in Bergey's Determinative Bacteriology (1948) the thermal death point of this virus is given as 42.5° to 75° C for 30 minutes. The virus of foot-and-mouth disease in defibrinated blood is rendered inactive in 20 minutes at 55° C, and in filtered, diluted vesicular fluid in 15 to 40 minutes, while at 60° C the vesicular contents are inactivated in 5 minutes, according to the British Commission (1927).

McCulloch (1945) found that 100,000 infective doses of fowl pox virus suspended in broth were inactivated in about 5 minutes at 60° C, in 15 to 20 minutes at 55° C, but the material resisted 50° C for longer than 1 hour. This investigator also stated that the sacbrood virus of bees is destroyed in 10 minutes at 59° C when suspended in water and in the same period of time at 70° to 73° C when suspended in glycerol or honey.

In a study involving six hospital outbreaks of lethal infantile diarrhea, Light and Hodes (1943) determined that the infectious agent, a filtrable virus, was wholly inactivated by boiling for 5 minutes but it resisted heating at 70° C for 1 hour. This infectious agent was also found to be attenuated upon prolonged drying or heating to 80° C for 1 hour.

The causative agent of infectious hepatitis (homologous serum jaundice) has been the subject of many investigations during the course of the last few years. Neefe (1946) reported that this virus, in human albumin, was inactivated by heating for 10 *hours* at 60° C. It is believed that this disease-producing agent is transmitted, in certain instances, by means of the single syringe-multiple injection method. The commonly used methods of sterilization of syringes, needles and lancets by brief exposure in boiling water or immersion in chemical solutions are recognized as inadequate for the destruction of this virus, particularly in the presence of organic substances.

In a study on the thermodestruction of a bacterial virus (bacteriophage against *Escherichia coli*) in water, Chang, Willner and Tegarden (1950) determined that the per cent survival of the virus ranged from 18.2 at 50° C for 60 minutes to 0.03 per cent at 70° C for 5 minutes. The death-rate constant was found to be independent of the initial concentration of the virus. At 65° C every 10-fold increase in virus concentration resulted in an increase of 26 minutes in exposure time to reach a complete killing of the virus.

The effect of heat as an inactivating agent on other forms of microbial life should also be considered but unfortunately space will not permit such a review. Of special interest, however, are the recent studies on the resistance of *Coriella burnetii* to physical and chemical agents by Ransom and Huebner (1951). This organism,

the causative agent of Q fever, was found to survive temperatures as high as 63° C when suspended in milk, sealed in vials and submerged for 30 to 40 minutes in water baths. Under similar conditions other rickettsiae such as *Rickettsia rickettsii* and *Rickettsia akari*, apparently did not survive 50° C for 15 minutes.

## SURGICAL STERILIZATION BY MOIST HEAT

*Steam Under Pressure.*—Moist heat in the form of saturated steam under pressure is the most dependable medium known for the destruction of all forms of microbial life. Because of this fact, steam under pressure is the universal standard for the sterilization of surgical supplies. Today, in every modern hospital, there may be found a variety of sterilizers or autoclaves, each performing a vital service in protecting the patient against infection, but all are dependent upon the application of certain fundamental principles allied with the use of steam as a sterilizing agent.

Steam is water vapor, and in the saturated state, it can exist only at a definite pressure corresponding to a given temperature. The term "saturated steam" means that the steam is at the maximum pressure and density possible at a given temperature. Saturated steam cannot undergo a reduction in temperature without a lowering of its pressure, nor can the temperature be increased except when accompanied by a corresponding increase in pressure. For sterilization processes steam under pressure is used rather than atmospheric steam for the sole purpose of attaining higher temperatures. Pressure of itself has nothing whatever to do with the microbicidal properties of steam. A scale of pressures and equivalent temperatures of saturated steam as employed for sterilization purposes is given in Table 110.

*How Steam Sterilization is Accomplished.*—Steam sterilization as normally conducted in the autoclave is a product of heat plus moisture in which the moisture factor plays an exceedingly important part. Steam gives up its heat in sterilizing only by the process of condensing back into the water from which it came. This means that every fibre or particle of any porous article undergoing sterilization will abstract or absorb (through condensation) an amount of moisture from the steam in exact proportion to the amount of heat absorbed by the article.

The condensation process of heating makes use of the latent heat of steam which within the normal range of sterilizing temperatures and pressures amounts to the liberation of about 520 calories per g of condensate. This factor is of tremendous importance in its application to the permeation of dry goods, fabrics or

TABLE 110.—SCALE OF PRESSURES AND EQUIVALENT TEMPERATURES OF SATURATED STEAM FOR STERILIZATION PURPOSES

<i>Pounds pressure</i>		<i>Temperature</i>		<i>Sterilizing application</i>
<i>Gauge</i>	<i>Abso- lute</i>	<i>Degrees F</i>	<i>Degrees C</i>	
80.3	95	324.1	162.2	Maximum pressure in steam supply lines. (For pressure sterilizers)
75.3	90	320.3	160.1	
70.3	85	316.3	158.0	
65.3	80	312.0	155.6	
60.3	75	307.6	153.0	Ideal pressure in steam supply lines.
55.3	70	302.9	150.6	
50.3	65	298.0	147.8	
45.3	60	292.7	144.8	
40.3	55	287.1	141.9	Minimum pressure in steam supply lines. (For pressure sterilizers)
35.3	50	281.0	138.3	
30.3	45	274.4	134.6	
27.3	42	270.2	132.3	
25.3	40	267.3	130.7	Emergency (high-speed) sterilization of instruments.
20.3	35	259.3	126.2	
19.3	34	257.6	125.3	
18.3	33	255.8	124.2	
17.3	32	254.1	123.4	Sterilization of hospital supplies—surgical in- struments, dressings, solutions, etc. Steriliza- tion of laboratory supplies. Commercial sterilization processes.
16.3	31	252.2	122.3	
15.3	30	250.3	121.2	
14.3	29	248.4	120.3	
12.3	27	244.4	118.0	Sterilization of laboratory supplies. Commercial sterilization processes.
10.3	25	240.1	115.6	
8.3	23	235.5	113.0	
6.3	21	230.6	110.3	
4.3	19	225.2	107.1	Terminal heating of infant formulas.
2.3	17	219.4	104.2	
0.0	14.70	212.0	100.0	
				Sanitization of instruments & utensils. Terminal heating of infant formulas. Streaming steam sterilization. Water boils @ sea level, New York City.
	14.13	210.	98.8	Water boils @ altitude 1025 ft., Omaha, Nebr.
	13.03	206	96.6	Water boils @ altitude 3115 ft., Calgary, Alta.
	12.01	202	94.4	Water boils @ altitude 5225 ft., Denver, Colo.
	11.06	198	92.2	Water boils @ altitude 7381 ft., Laramie, Wyo.
	10.40	195	90.5	Water boils @ altitude 9000 ft., Quito, Ecuador
	8.95	188	86.6	Water boils @ altitude 12,700 ft., LaPaz, Bolivia

textiles. For example, as steam contacts the outer layer of fabric, the cooler substance immediately causes a film of steam to condense leaving in the fabric a minute amount of water. The next film of steam immediately fills the space created by the volume collapse of



the previous film but it does not condense in this outer layer, rather it passes through and attacks the second layer of fabric—condenses and heats it. So on until the entire mass has been heated, after which the package will contain an amount of moisture (condensate) exactly equivalent to the amount of heat abstracted from the steam.

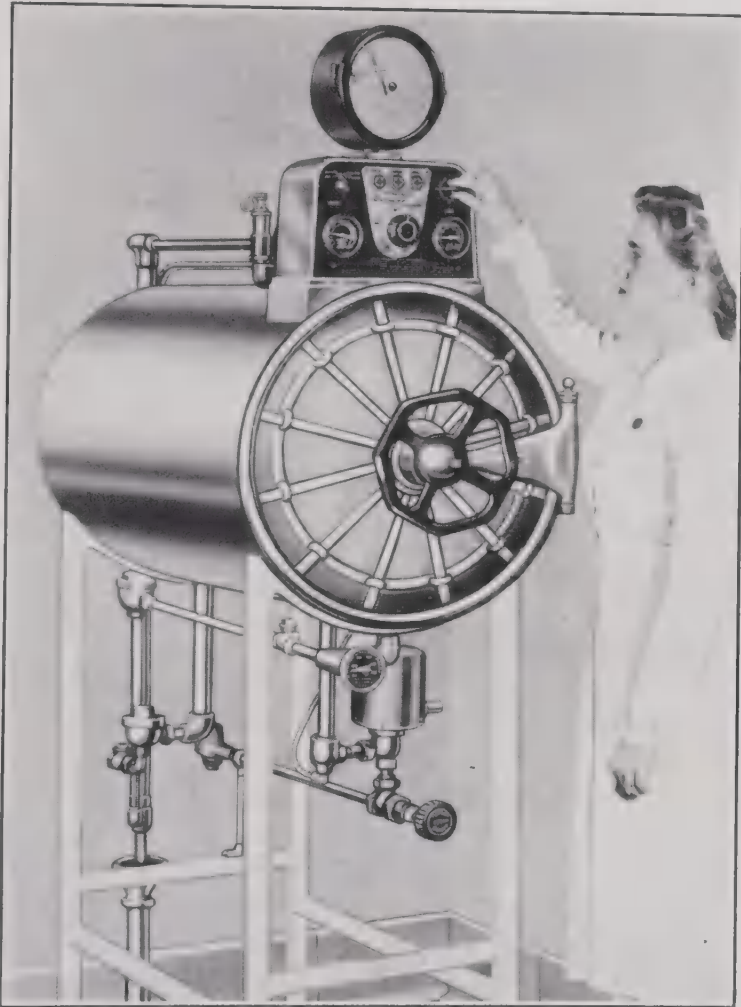


Fig. 25.—A modern surgical supply sterilizer, cylindrical type, direct steam heat, equipped with "Cyclomatic" Control for automatic operation of all phases of the sterilizing cycle. (Courtesy of American Sterilizer Company.)

Continuation of the process will cause no further condensation, but the temperature of the mass will remain constant at the temperature of the surrounding steam.

Since all steam sterilization is based upon "direct steam contact," it follows that the same process of condensation and heating applies to instruments, utensils or other articles undergoing surface

sterilization. With such materials, there is no permeation with steam through the metal, the object being only to heat and sterilize the surface. In this case the cold metal condenses the steam until the article is heated to the temperature of the steam. Throughout sterilization the metal surfaces are bathed with an abundance of moisture as compared with porous fabrics, which greatly facilitates sterilization. Because of the rapid heating effect and the abundance of moisture, it becomes possible to prescribe a shorter period of exposure for instruments than for fabrics which require time for permeation.

TABLE 111.—STERILIZER TEMPERATURES WITH VARIOUS DEGREES OF AIR DISCHARGE

Gauge pressure pounds	Sat'd steam complete air discharge		Two-thirds air discharge (20" vacuum)		One-half air discharge (15" vacuum)		One-third air discharge (10" vacuum)		No air discharge	
	° C	° F	° C	° F	° C	° F	° C	° F	° C	° F
5	109	228	100	212	94	202	90	193	72	162
10	115	240	109	228	105	220	100	212	90	193
15	121	250	115	240	112	234	109	228	100	212
20	126	259	121	250	118	245	115	240	109	228
25	130	267	126	259	124	254	121	250	115	240
30	135	275	130	267	128	263	126	259	121	250

*The Modern Surgical Supply Sterilizer.*—In figure 25 is shown a typical steam heated pressure steam sterilizer of the surgical supply type, equipped with "Cyclomatic" Control for automatic operation. The structural and valving features as provided by different manufacturers will vary to some degree, but the essential elements of control are the same. Although this sterilizer is properly termed a "pressure steam sterilizer," its performance is gauged not by pressure but by temperature as measured by a thermometer. Pressure gauges are provided, one for the jacket that surrounds the chamber and another for the chamber in which supplies are sterilized. However, these gauges are of minor significance because the sterilizing power of steam is a function of its temperature rather than its pressure. Also, pressure is not necessarily significant of the temperature of steam. For example, saturated steam free from air at 15 pounds gauge pressure will result in temperature of 121° C (see Table 111). But if no air is evacuated from a chamber and steam is admitted until the gauge registers 15 pounds, the maximum temperature attained after the steam and air have mixed will be only 100° C (212° F), as shown in figure 26. This is a typical application of Dalton's law of partial pressures. Thus it may be seen that air evacuation from the sterilizing chamber is a fundamental requirement for effective sterilization.

In figure 27 is shown a longitudinal cross-section of the same sterilizer as illustrated in figure 25. The body of the sterilizer consists of the cylindrical sterilizing chamber surrounded on the sides by a steam jacket (steam space) which is enclosed by the

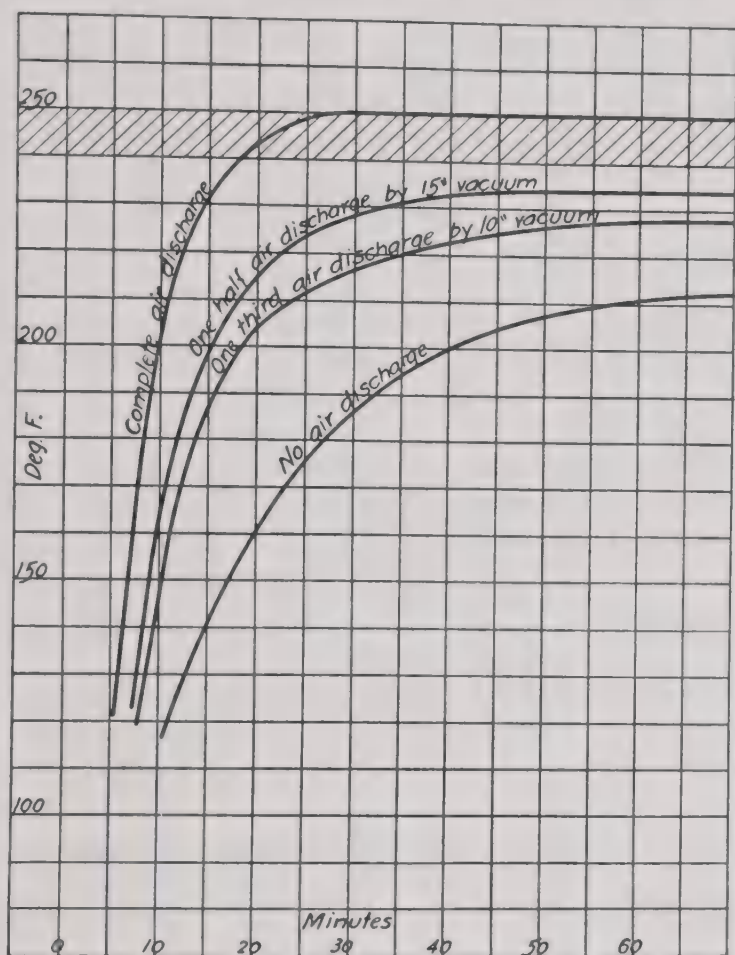


Fig. 26.—The temperatures resulting from incomplete evacuation of air from a dressing sterilizer. (Courtesy of American Sterilizer Company.)

outer shell covered on the sides by a finishing jacket. A safety steam-locked door closes the front end of the chamber and it is made steam tight by compression through the door mechanism against a flexible gasket. Steam from the main supply line or source is admitted at the bottom of the steam jacket. In this diagram, the sterilizer is heated by direct steam from the supply line and the pressure is regulated to the desired range by initial adjustment of the pressure regulator. If the sterilizer is heated by electricity or gas, a steam generator or boiler is mounted under the sterilizer, and steam is taken directly to the jacket from this source.



Control of pressure then is governed by automatic regulation of the heat.

In beginning operation with a cold sterilizer, steam is first admitted from the source to the jacket, with the connection to the chamber closed, until the jacket pressure becomes constant at 15 to

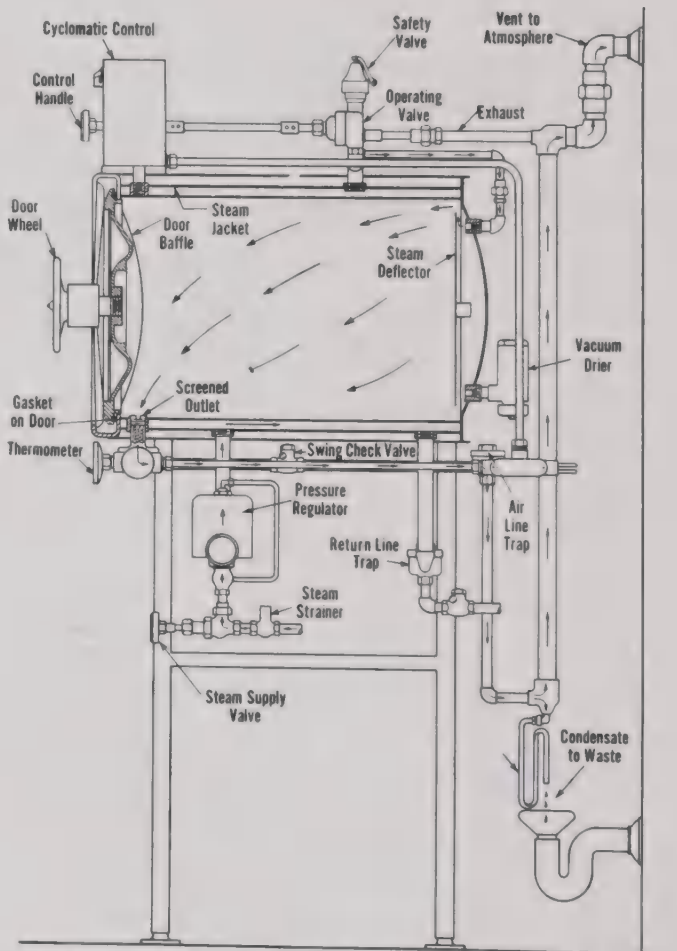


Fig. 27.—Longitudinal cross-section of sterilizer shown in figure 25. The arrows indicate the flow of steam from the source (supply line) through the various operating parts of the sterilizer. (Courtesy of American Sterilizer Company.)

17 pounds. This constitutes the reservoir from which the chamber steam will be drawn. In sterilizing, the load is placed in the chamber and the door locked while the jacket is heating. Then, with the jacket pressure stabilized, the operating valve is turned to "Sterilize" which permits the jacket steam to flow through the multi-port valve, through the piping on top of the sterilizer and into the chamber at the top center of the back end. Here the steam is deflected by means of a baffle to prevent undue wetting of the load.

It should be remembered that when steam is admitted to the chamber, the chamber is filled with air and also the more or less porous supplies. This air must be evacuated in order that the proper temperature can be promptly attained, and to facilitate thorough permeation of the load with steam. That evacuation is accomplished as follows:

Air is more dense than steam, and as steam enters the chamber it gravitates above the air, fills the upper areas of the chamber and compresses air at the bottom. Since steam is admitted under pressure, air in the lower areas is forced out through the screened outlet at the extreme bottom near the front end, through the thermometer case and the pipe that leads to the thermostatic (air line) trap, then on to the vertical pipe which is vented to the atmosphere at the top and drained to the waste through the open (sanitary) funnel at the bottom. This air break in the connection to the drain is highly important. It prevents possible back flow of contaminated waste to the sterilizer. When the sterilizer is cool, the thermostatic (air line) valve is open, offers no restriction to the flow of air and condensate to the vent. Only when air evacuation is complete and steam finally flows to the thermostatic valve does it close off, interrupting the discharge. Thereafter it will open intermittently to discharge condensate as it accumulates. This method of air evacuation is known as the "gravity" system and is almost universally used on pressure sterilizers that have been produced since 1933.

It is obvious from the foregoing that there must be some method for definite measurement of the degree of air discharge and quality of steam in the chamber. This is the specific function of the thermometer which, located in the discharge line that drains air and condensate from the chamber, will immediately respond to any interruption of air discharge from the chamber. Under any condition of performance, the thermometer will indicate never less than the temperature of the coolest medium with which the load is surrounded. This is true because air or any mixture of steam with air will gravitate below pure steam. The thermometer, therefore, when properly located is a reliable means of measuring the true sterilizing quality of the steam that is contacting the load. When the sterilizer is functioning correctly, temperature will advance within 5 to 10 minutes to 121° C at which point the period of exposure is timed. When the exposure period is complete, steam is exhausted from the chamber, leaving jacket pressure maintained at the same range, by turning the operating valve to the "Exhaust" position. The exhaust steam escapes through the venting system.

*Minimum Standards for Surgical Sterilization.*—In establishing minimum standards for surgical sterilization, careful attention should be given to the prescribing of exposure periods for the various types of loads that will insure a time-temperature relationship adequate for the destruction of the most resistant forms of microbial life. In other words, it is expedient always to prescribe a performance which carries a reasonable factor of safety in terms of temperature or time or both, based upon reliable experimental data relating to the destruction of the pathogenic spores, but great enough to provide for the destruction of the still more resistant non-pathogenic spore-bearing organisms. The oft quoted statement that “no living thing can survive 10 minutes direct exposure to saturated steam at  $121^{\circ}\text{C}$  ( $249.8^{\circ}\text{F}$ )” would appear to be a reasonably accurate standard based upon recorded literature data. However, Bigelow and Esty (1920) reported the existence of thermophilic spores which required 23 minutes of direct exposure to saturated steam at  $120^{\circ}\text{C}$  for their destruction. Similarly, Black and Tanner (1928) found that certain aerobic thermophiles survived  $100^{\circ}\text{C}$  for 24 hours,  $115^{\circ}\text{C}$  for 1 hour, and  $120^{\circ}\text{C}$  for 25 minutes. In the majority of instances, closely checked experiments have indicated that most resistant bacterial spores can rarely withstand five minutes’ exposure to saturated steam at  $121^{\circ}\text{C}$ , while apparently none of the pathogenic organisms have been shown to be resistant to an exposure of even 3 minutes. (Table 112).

In developing a safe minimum standard for surgical sterilization, several workers have proposed the use of soil which contains a variety of aerobic and anaerobic spore-bearing and non-spore-bearing organisms as a control. Ecker (1937), for example, determined that 1-gram samples of air dried and powdered garden soil inserted in the center of laparotomy sets, and maternity supplies, were found to be sterile after exposure at  $115^{\circ}\text{C}$  in the autoclave for periods of 60, 45, 30 and 15 minutes. When samples of the same soil were placed in carefully packed dressing drums it was found that an exposure of 45 minutes at  $115^{\circ}\text{C}$  was necessary for sterilization.

Most authorities are in agreement that in direct contact with saturated steam at a temperature of  $121^{\circ}\text{C}$ , a period of 5 to 10 minutes is sufficient to insure destruction of most resistant forms of microbial life. However, to clearly define minimum standards of time and temperature required for sterilization of the wide variety of surgical supplies is difficult. Not only must the relationship selected be bacteriologically safe, but it must also permit the prescribing of practical exposure periods for the various supplies, including suitable margins of safety, with due consideration for



TABLE 112.—TYPICAL DESTRUCTION TIMES (IN MINUTES) OF BACTERIAL SPORES SUBJECTED TO MOIST HEAT

Organism	Investigator									
	100° C 212° F	105° C 221° F	110° C 230° F	115° C 239° F	120-121° C 248-250° F	125° C 257° F	130° C 266° F	135° C 275° F		
B. anthracis	2								Schneiter & Kolb (1948)	
B. anthracis	5-15								Stein & Rogers (1945)	
B. anthracis	5-10	5-10							Murray (1931)	
B. cereus	6								Schneiter & Kolb (1948)	
B. subtilis	6-17								Schneiter & Kolb (1948)	
B. subtilis	10								Ecker (1937)	
Cl. botulinum	330	100	32	10	4				Esty & Meyer (1922)	
Cl. botulinum			>30	10	4				Hoyt, Chaney & Cavell (1938)	
Cl. botulinum	300	120	90	40	10				Tanner & McCrea (1923)	
Cl. botulinum	300	40			6				Weiss (1921)	
Cl. oedematiens			10	4	1				Hoyt, Chaney & Cavell (1938)	
Cl. oedematiens			15						Ecker (1937)	
Cl. septicum			5						Ecker (1937)	
Cl. tetani	5-15	5-10							Murray & Headlee (1931)	
Cl. welchii	5-10								Headlee (1931)	
Cl. welchii		5							Ecker (1937)	
Putrefactive anaerobe 367 <sup>a</sup>	780	170	41-6	15-6	5-6				McCulloch (1945)	
Thermophiles	834	405	100	40	11-12	3-9-4-6	1-7-2-2	0-7-0-9	Bigelow (1919) (1921)	
Soil bacilli	>660			15					Ecker (1937)	
Soil bacilli	>1020	420	120	15	6	4	1	0-5	Konrich (1938)	

economic factors. Carefully conducted studies strengthened by experience in many hospitals have shown that minimum standards of time and temperature substantially greater or less than the following are either incompatible with modern sterilizer design and unnecessarily destructive of materials and supplies or unsafe from the standpoint of effective sterilization:

<i>Degrees C.</i>	<i>Degrees F.</i>	<i>Time (minutes)</i>
132	270	2
125	257	8
121	250	12
118	245	18
115	240	30

The above times and temperatures should not be interpreted as denoting prescribed periods of exposure for the various types of surgical supplies. Rather, they indicate minimum time-temperature ratios to be maintained throughout all portions of a load in order to accomplish effective sterilization. They do not take into account the additional time factor required for steam penetration of porous supplies or the rate of heat transfer through solution containers.

There is little justification for maintaining steam temperature higher than 121° to 123° C, equivalent to 15 to 17 pounds pressure, because sterilization occurs within a brief period at this range. Another reason for limiting the temperature to this range is that it has been demonstrated repeatedly that 121° C is the critical temperature for most surgical supplies. Exposure to temperature materially beyond this point brings about more or less speedy destruction of fabrics, rubber goods, etc., and is wasteful of time and fuel.

There is also the mistaken supposition that steam at the higher pressures, in excess of 20 pounds, permeates the load much faster than steam at 15 pounds pressure. To be sure, the higher pressure will result in a correspondingly higher temperature in any modern sterilizer, but the rate of heating to the desired range of 121° to 123° C is not sufficiently greater to justify serious consideration. It is more essential to be certain that a properly designed sterilizer is operated efficiently; that air is removed from the chamber; that the temperature of the discharge line is relied upon for the degree of heat attained; and that the materials to be sterilized are correctly prepared and loaded in the sterilizer, rather than to be careless about these essentials and attempt to compensate by long periods of exposure at higher than necessary steam pressures.

*Recommended Periods of Exposure.*—Reliable routine steam sterilization of surgical supplies can be accomplished through the

integration of four factors, namely (1) regulation of the sterilizer so as to maintain a temperature of 121° to 123° C (250 to 254° F), equivalent to 15 to 17 pounds pressure; (2) correct methods of packaging; (3) proper loading of the sterilizer; and (4) period of exposure. With intelligent application of these requirements the following exposure periods should provide ample time for heat penetration and sterilization:

	<i>Minutes</i>
Surgical packs, normal size in muslin covers . . . . .	30
Dressing drums, with muslin liners . . . . .	45
Instruments in trays, with muslin covers . . . . .	15
Instruments, wrapped for storage . . . . .	30
Utensils in muslin covers . . . . .	15
Rubber gloves in muslin wrappers . . . . .	20
Treatment trays with muslin wrappers . . . . .	30
Dressing jars, loosely packed, on sides . . . . .	30
Glassware, empty, inverted . . . . .	15
Syringes, unassembled, in muslin or paper covers . . . . .	30
Sutures, silk, cotton or nylon . . . . .	15

## STERILIZER CONTROLS

*Recording Thermometers.*—When properly applied and used, the recording thermometer is a practical detector of faulty sterilization performance. This instrument (Fig. 28) contains a standard clock mechanism, electrically operated or mechanically wound, which revolves an 8-inch diameter chart once in 24 hours. It indicates and records the same temperature as that shown by the indicating thermometer located in the discharge system of the sterilizer chamber, and it also records the duration of each exposure. Lacking the recorder, the operator can, and not infrequently does, forget to time the performance when the temperature has advanced to 121° C as prescribed. The instrument does not forget—and the operator can quickly see when the period of exposure is over. It also furnishes the supervisor with a reliable record of what has transpired during the day. If the periods of exposure are greater or less than prescribed, or if the temperature has not been maintained within the proper limitations there is a positive record of the errors, thus providing evidence needed upon which to act in correcting discrepancies.

Recording thermometers are subject to some distortion of the highly flexible pen arms but means for readjustment are provided. Accuracy of the recorder should be checked at weekly intervals or oftener as follows: While sterilizing, when the temperature has become stable at the maximum range, check the recorder with the indicating thermometer located in the chamber discharge line of



the sterilizer. If there is any difference adjust the screw on the pen arm until the temperature of the recorder is the same as that shown by the indicating thermometer. In the author's opinion, this instrument should be considered a necessary part of the equipment for every general sterilizer because its proper use most certainly

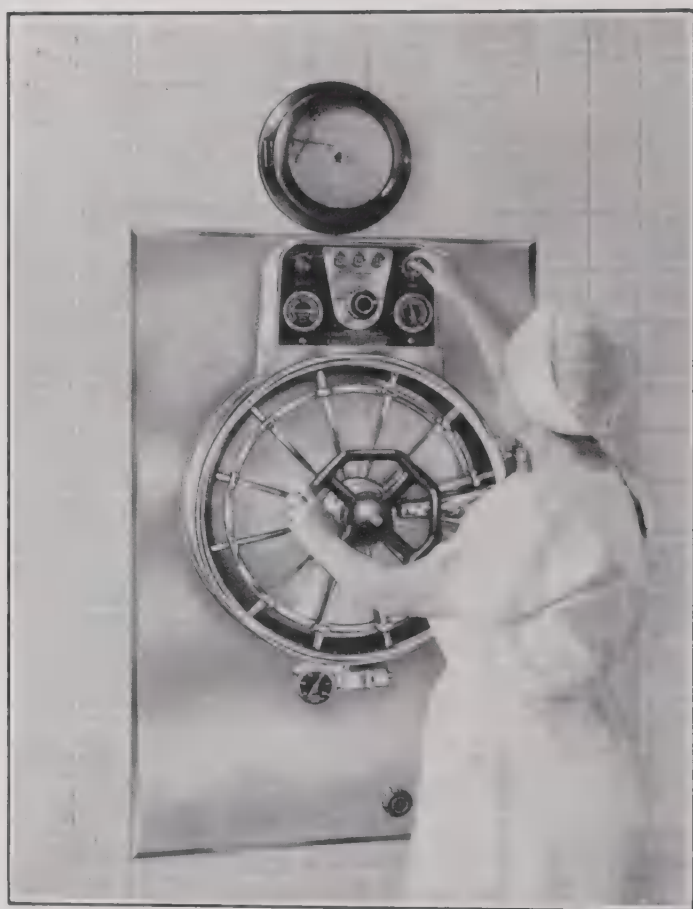


Fig. 28.—The recording thermometer mounted on the wall above the automatic control provides a continuous record of each sterilizing cycle. Instruments of this type promote safer sterilizer performance and assist in maintaining established standards. (Courtesy of American Sterilizer Company.)

promotes safer performance. The ability to prove with daily chart records that definite standards of timing and temperature are being maintained should appeal to those who must shoulder the responsibility for sterilization.

*Indicating Potentiometers.*—The most comprehensive method for testing the functional efficiency of sterilizers, the penetration of steam through a porous load or the rate of heat transfer through

solution containers involves use of a potentiometer, an instrument for measuring temperature in the most inaccessible portion of the load during sterilization. When using this instrument, access to

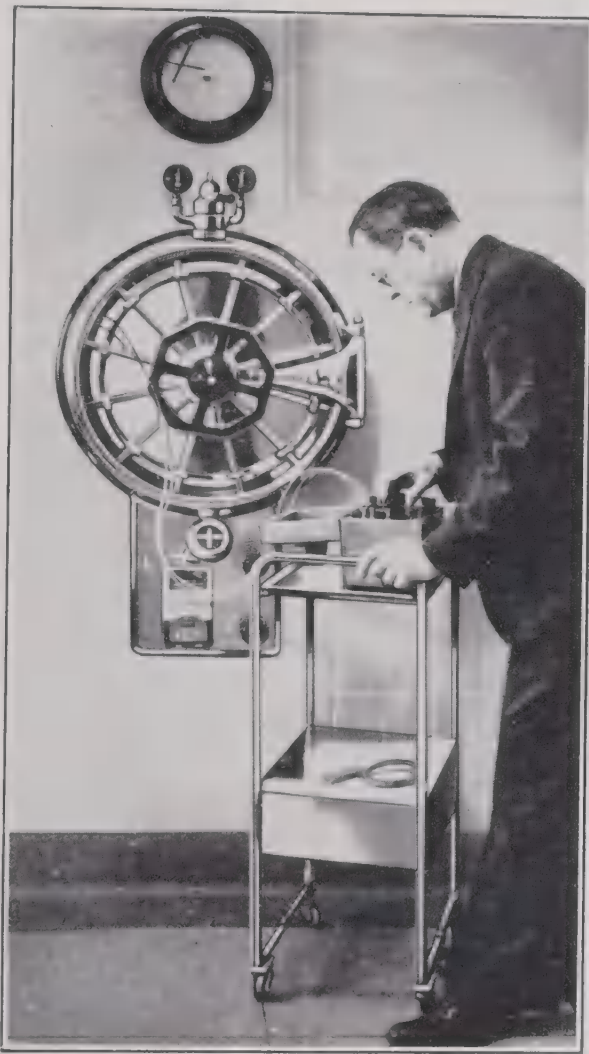


Fig. 29.—The potentiometer and thermocouple may be used to excellent advantage in testing the functional efficiency of sterilizers or in determining the rate of heating of a given load. The thermocouple leads are inserted under the edge of the door and the point is located in the center of the test pack. (Courtesy of American Sterilizer Company.)

he load is gained through a thermocouple, two small wires of different metals (usually copper-constantan) welded together at the ends and inserted in the load. The terminal ends are brought out under the door of the sterilizer, impinging against the door gasket, and connected to the potentiometer, as in figure 29. Any change in temperature at the joined ends of the couple is immediately indicated

on the dial of the instrument which normally has an accuracy of adjustment of the order of  $\pm 0.25^{\circ}\text{C}$ . In this manner, the penetration of steam through a load can be followed, readings being taken at frequent intervals and the results then plotted on graph paper. With such charts one can easily determine if a given cycle of performance provides a suitable factor of safety.

In using the potentiometer, the chamber of the sterilizer should be filled to the maximum capacity contemplated for routine loading. The pack selected for observation should be the largest and most densely arranged one and it should be located near the bottom of the chamber to which point the steam will travel with the greatest lag. The thermocouple point should be buried in the interior in the most compact part of the pack to which steam will gain access most slowly. If the preparation of the packs has been standardized and if the loading of the chamber is typical of routine practice, such tests are highly significant.

For bulk loads of surgical packs, both large and small, 30 minutes' exposure at  $121^{\circ}$  to  $123^{\circ}\text{C}$  should show a reasonable margin of safety in time beyond that required to meet the minimum standard. For example, if a test pack showed that 12 minutes were required to reach  $121^{\circ}\text{C}$ , measured from the time the discharge line thermometer reached this temperature, it would require an exposure of 24 minutes to meet the minimum standard. To this should then be added an additional 6 minutes for a fair margin of safety, making a total of 30 minutes' exposure. If the pack is so dense that the minimum standard cannot be maintained in 30 minutes' exposure, the correct procedure would be to revamp the package, perhaps remove some of the more compact articles and wrap those separately. Sterilization should not continue for much longer than 30 minutes, because added exposure becomes increasingly harmful, especially to the outer layers of fabrics.

*Automatic Time-Temperature Controls.*—During the past few years sterilizer manufacturers have made a definite contribution toward advancement of the art through development of automatic control mechanisms designed to minimize the human element in sterilizer operation. Typical of the class of automatic time-temperature controls is the instrument shown in figure 28, mounted on top of the sterilizer. Its primary function is not, however, limited to automatic timing of the selected period of exposure, but rather it extends to all phases of the sterilizing process, to the end that all steps in the cycle normally carried out manually by the operator are conducted automatically according to a predetermined pattern. This means that when the control is set for operation, it automatically times the period of exposure at the correct temperature,



exhausts the steam from the chamber, governs the process of drying, and, finally, sounds an alarm announcing the completion of the cycle. The mechanical means responsible for this sequence of operations is a motorized multi-port valve located on top of the sterilizer in back of the instrument control case. The feature of automatic time-temperature control is accomplished through the use of a highly sensitive and accurate thermoswitch located in the chamber discharge line, adjacent to the indicating thermometer. The thermoswitch, in turn, is connected to an automatic reset timer by means of wiring and conduit leading upwards from the chamber drain line to the rear of the control box on top of the sterilizer.

In discussing the functional efficiency of automatic controls in pressure steam sterilization, it should be understood that any such control device must, of necessity, have certain limitations because it cannot indicate or compensate for faulty methods used in the preparation, packaging and loading of supplies in the sterilizer. In short, regardless of the perfection of the sterilizer and the controls to guarantee unvarying uniformity of operation, sterilization of the load is still dependent upon correct methods of packaging and strict adherence to established rules for loading the sterilizer.

The prime purpose of any automatic control is to guard against the inaccuracies of human behavior in sterilizer operation. To this end, it is obvious that the instrument should eliminate the mental burden of remembering the time at which the exposure period began and when it should end. Likewise, it should eliminate errors in the reading of the thermometer, insure the proper sequence of each step in the sterilizing cycle and protect the load against under-exposure or over-exposure. The reliability of the control should merit complete confidence from the operator to the extent that once it is set in operation, the exact period of continuous exposure at the correct temperature, as well as the entire cycle of performance, will be carried out with accuracy and precision not obtainable through manual operation. The use of automatic controls naturally presupposes the need for periodic inspection and servicing to insure peak efficiency of operation. A properly trained mechanic or serviceman familiar with the equipment should always be engaged for this work. If these requirements are met, sterilizers equipped with automatic controls can be depended upon to afford a greater factor of safety in sterilization with a substantial saving in personnel time, materials and supplies.

## STERILIZATION INDICATORS

The subject of sterilization indicators has long been a controversial issue. Authoritative opinion is divided on the actual worth

of all sterilization controls or indicators of the "tell-tale" variety. Ecker (1937), for example, in evaluating the efficiency of these devices found that their turning points, based on color changes, or the melting of the indicator substance, had not been standardized and they varied in the time-temperature ratios required for complete change. In contrast to these findings, Hoyt (1934) reported that one type of indicator, when properly used and interpreted, was found to be an adequate check on the efficacy of sterilization of rubber gloves. On the basis of hundreds of tests under widely varying conditions of application, Underwood (1941) reported that the hermetically sealed glass tube control reacted (melted) uniformly when subjected to temperatures of 120° to 122° C for 5 to 8 minutes. At slightly lower temperature (118° C) the time required to fuse the control ranged from 20 to 30 minutes. These results encouraged Underwood to make the recommendation that approved sterilization indicators can be used to excellent advantage as an occasional check against faulty packaging, loading and performance of sterilizers. An evaluation of a group of tell-tale indicators by Walter (1937) revealed marked discrepancies in individual controls with a sufficient number showing delayed changes in end points so as to confuse operators and to cause unnecessary resterilization of supplies.

It is this writer's belief based upon observations over a period of years that all sterilization indicators possess the same general disadvantage, to a greater or lesser degree, in that a percentage will be found to react to a time-temperature ratio inadequate for sterilization or that the end points are not sufficiently clear so as to permit accurate interpretation of the results. Furthermore, the commercial sterilizer controls do not indicate the actual build-up of temperature in the test pack nor do they indicate how much over-exposure may have been applied. Manufacturers of such controls have not attempted to bring about uniformity or standardization of end points to conform to a safe time-temperature relationship required for sterilization of supplies. Consequently one type of indicator used in one hospital may react to a different time-temperature relationship than another type employed in another hospital.

If sterilization indicators are to be used, the indicator of whatever form it may be, should be placed in the center of the largest and most densely wrapped package in the load. This package should then be placed on the tray or shelf in the bottom of the sterilizer chamber, as in figure 30. When indicators are used for setting up standards for sterilizing systematically prepared loads, a heavy load of supplies as large and dense as any that will be encountered in routine practice should be assembled. Then select six of the

heaviest and most densely wrapped packs or drums. Place one indicator or control in the center of each of these packs, then place the packs on edge in the bottom of the sterilizer. Add the remainder of the load as it will normally be placed. The load should then be subjected to a 30-minute exposure, timing the period when the thermometer indicates  $121^{\circ}\text{C}$ , with the sterilizer regulated to produce a maximum of  $121^{\circ}$  to  $123^{\circ}\text{C}$ . Upon completion of the cycle, remove the load and examine the controls.



Fig. 30.—This diagram illustrates the correct and incorrect use of sterilization indicators. Control (1), in the upper part of the sterilizer, even though it is located in the center of the packs, serves no useful purpose. The sterilizing temperature would be attained in this pack quite rapidly—long before similar temperature could penetrate to the lower areas. Controls (2) and (3) are also useless because steam would contact them almost instantly. Controls (4) and (5) only are indicative of proper application. They are located in the centers of the large packs in the most inaccessible and coolest part of the sterilizer. (Courtesy of American Sterilizer Company.)

*Culture Tests.*—The use of culture tests to evaluate the effectiveness of a sterilizing process is usually the method of choice. Certainly they constitute a more direct approach to the lethality of the process and they are more reassuring to bacteriologists and surgeons in general. Unfortunately, the delay entailed in determining the results of culture tests makes them generally impractical except for the occasional check on the maintenance of minimum standards. If they are not properly planned they may be distinctly misleading.

For those who desire to employ culture tests the method outlined by Ecker (1937) is recommended. This procedure involves the use of air dried and powdered garden soil (1-gram) samples in paper packages inserted in the center of test packs, drums, etc.



In hospitals with properly equipped laboratories and trained technicians a periodic check of sterilization with the aid of soil samples can be easily performed. The soil test will give a safe biological index of sterility.

*Factors Contributing to Sterilization Failures.*—Inadequate sterilization occurs in many hospitals even though personnel charged with this responsibility have instructions to expose the surgical supplies to saturated steam at 121° C for 30 minutes. As a rule, surgeons recognize that all instruments and supplies used in the performance of an operation constitute a potentially major source of contamination for operative wounds. Dandy (1932), for example, has attributed the majority of postoperative infections to inadequate sterilization of the towels, gowns, gauze and other supplies that pass through the sterilizers. Inasmuch as he reported the infectious organisms to be either streptococci or *Micrococcus pyogenes* var. *aureus*, this would indicate an almost inconceivably low degree of efficiency in the steam sterilizers or an extremely high degree of carelessness on the part of the operators, because these organisms are not unusually resistant to heat.

For the most part, sterilization failures are the result of a series of factors, either singly or combined, which make up the human equation in sterilizer operation. Those factors of principal importance are:

(a) Failure to observe and understand the regulation of the sterilizer so as to maintain a pressure of 15 to 17 pounds, equivalent to 121° to 123° C.

(b) Incorrect methods of packaging and wrapping of supplies with no regard for the size and density of the individual packs.

(c) Carelessness in loading the sterilizer, with disregard for the necessity of providing for complete air removal and for free circulation of steam throughout the load.

(d) Failure to time correctly the required period of exposure—usually due to ignorance or negligence on the part of the operator.

(e) Failure to carry out the correct sequence of operations in the sterilizing cycle, as the result of carelessness, fatigue or distraction.

(f) Attempts to sterilize materials which are impervious to steam, such as talcum powder and petrolatum.

(g) Faulty equipment and lack of basic knowledge concerning the principles of operation and care of sterilizers.

*Pressure Gauge Reading.*—One of the more common errors responsible for sterilization failures is reliance on the pressure gauge reading rather than the thermometer as the true index of the sterilizing process. In spite of all that has been written on this

subject during the last 50 years the tendency still prevails to think of steam sterilization in terms of pressure rather than temperature. Moist heat is the sterilizing agent. Pressure is only incidentally significant. A steam pressure of 15 pounds (gauge) at sea level, with an additional pound added for each 2000 feet of elevation, can mean an internal sterilizer temperature of  $121^{\circ}\text{C}$ . It likewise can mean any lower temperature down to  $100^{\circ}\text{C}$ , depending upon defects in design and repair of the sterilizer and degree of improper operation.

The pressure gauges as commonly used on sterilizers are inaccurate, even when adjusted so the hand returns to zero when the sterilizer is idle. The so-called commercial tolerance at the sterilizing range of 15 to 20 pounds pressure is between 1 and 2 pounds. This means that the reading of the gauge at these pressures may vary 1 or 2 pounds on either side of the indicated pressure. For this reason, safe practice demands that the thermometer located in the chamber discharge line be used as the indicator of the degree of moist heat in the sterilizer. A thermometer placed near the top of the chamber will naturally receive the maximum benefit of the steam, but one placed in the chamber discharge line will be much more reliable in indicating inefficient sterilization.

*Air Elimination.*—When steam under pressure is admitted into a sterilizing chamber containing air, the steam will gravitate to the top of the chamber, compressing the air at the bottom. Air and steam are reluctant to mix. In fact, they do not mix until the air can gradually absorb part of the heat from the steam. The period required for this mixture to occur is most uncertain and it can only be determined by actual temperature measurements.

Since air has no bactericidal effect, and the sterilizing efficiency of steam under pressure depends upon moisture and heat, a small amount of entrapped air may lead to sterilization failure. The effect of entrapped air upon the temperature attained by materials placed in different locations in the sterilizer is shown in figures 26 and 31. The low rate of heating is due to the fact that saturated steam heats by transfer of its latent heat as it condenses on the surface of a cooler object and the rate of condensation is materially lessened by the presence of air.

The majority of sterilizers or autoclaves manufactured today employ the gravity process for the elimination of air from the chamber as discussed on page 669. Sterilizers which employ the steam ejector vacuum system, as a means of air elimination, fall far short of the requirement for accurate, dependable work. It is extremely difficult with any known practicable device, such as an ejector valve, to create a vacuum of sufficiently high degree to

warrant its use. The temperatures resulting from incomplete air evacuation are not satisfactory for sterilization as evidenced in Table 111.

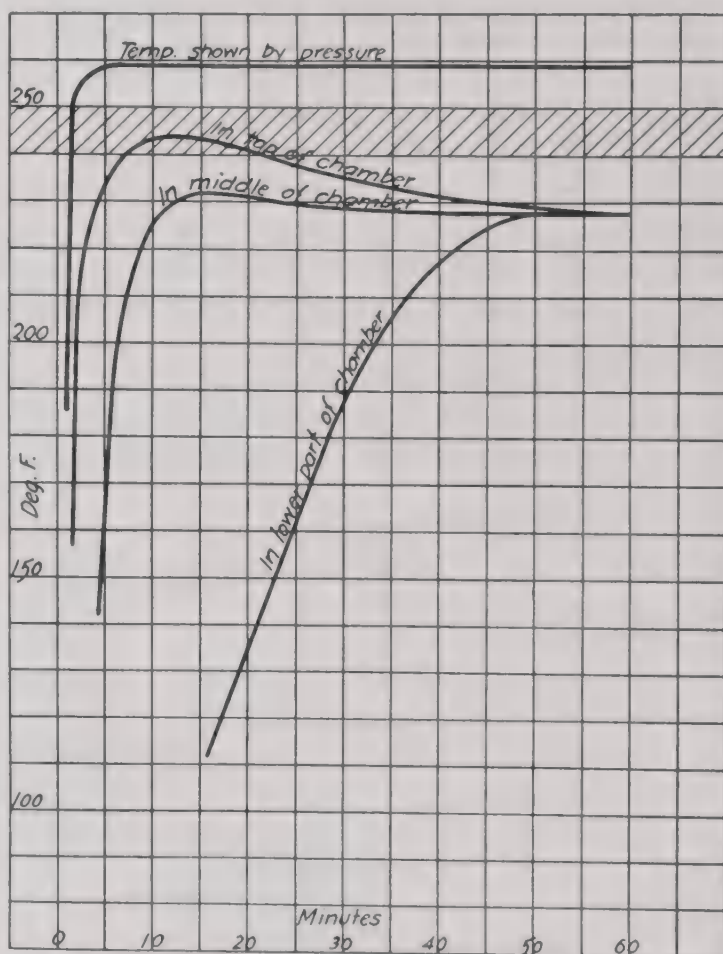


Fig. 31.—The above curves show the radical differences in temperature which may be found in different parts of a sterilizing chamber from which air has not been evacuated. The same relative conditions will apply if only part of the air remains in the chamber. (Courtesy of American Sterilizer Company.)

## PREPARATION OF SUPPLIES FOR STERILIZATION

*Surgical Packs.*—In preparing supplies for sterilization and in establishing correct methods of packaging, operators should constantly bear in mind that safe performance demands complete permeation of every strand and fiber of the materials with the moisture and heat of the steam. This permeation will occur rapidly or slowly, depending upon the size and density of the packs and upon the arrangement of the load in the sterilizer. If the individual pack is



too large and dense, so much time will be required for the steam to penetrate to the interior that the outer portions will be over-sterilized and, since the exposure period must be sufficient for the largest packs, all the smaller packs will be over-sterilized, perhaps harmfully.



Fig. 32.—This major lap pack, arranged for muslin wrapping, is suggested as a model for all heavy packs. (Courtesy of American Sterilizer Company.)

The first requirement is, therefore, to restrict the size and density of the individual pack so that 30 minutes' exposure will insure uniform steam permeation with an adequate margin of safety in sterilization. The practice of using large dense bundles involves too many hazards. The largest pack should not exceed 12"x12"x20" in size for routine work. When tests indicate that any pack, sterilized by itself, requires more than 30 minutes' exposure, that indicates the pack is too large or too dense and it should be broken down into two or more smaller packs.

The major pack shown in figure 32, arranged for muslin wrapping, is suggested as a model for all heavy packs. The materials are arranged so as to promote rapid and complete permeation of steam through the mass. The pack includes:

22 hand towels	1 tongue depressor
2 skin towels	4 applicators
1 lap sheet	24 sponges 4" × 4"
1 Mayo table drape	12 sponges 4" × 8"
1 cautery cover	6 large abdominal sponges
	1 package tonsil sponges

To facilitate work when this pack is opened in the surgery, the table drape is folded once only and spread out on the work table in two thicknesses only. It is used to form the inner covering of

the pack. The gauze sponges are located near the center of the pack to break up close contact between masses of other more closely woven fabrics. Other articles are all folded flat and each succeeding layer is placed crosswise of the one below, to promote free circulation of steam through the mass. When the materials are all assembled as shown, the extended sides of the table drape are used to cover the pack, then a double thickness muslin cover is applied. The covers should not be drawn up too tightly, only enough to hold the materials together. The outer cover can be secured with tie strings or with a No. 1 awning cord. Pins should not be used because they invite contamination.

*Protective Covering for Surgical Supplies.*—In recommending a material to serve as a protective cover or wrapper for surgical supplies one should bear in mind that the cover must provide protection against contact contamination in handling and it must also serve as a dust filter. When the load is removed from the sterilizer all porous supplies are more or less filled with vapor and as this vapor condenses it tends to create a partial vacuum within the goods which results in a definite intake of room air from which dust particles must be removed in the covering envelope or the contents may be contaminated. Wide experience has shown that two thicknesses of good quality (unworn) muslin serves this purpose practically and effectively to the extent that packs so covered will remain sterile in clean storage for as long as two weeks, after which they should be resterilized.

Canvas covers should never be used because the tightly woven fabric seriously retards the passage of steam and interferes with sterilization. For the same reason canvas should not be used for table covers or any other requirement where sterilization is necessary. Cellophane is also unsuitable as a wrapping material because it is impervious to steam. If the steam transfer characteristics of a material are unknown then they should be evaluated before placing it in use for the preparation of surgical supplies.

In general, paper wrappers for surgical supplies are not recommended, principally because of the possibility of rupture in handling. Coarse, brown wrapping paper (40 lb Kraft) will pass steam quite freely and, provided there are no holes in it, will also filter out dust particles satisfactorily. The principal objection to its use for surgical supplies is based upon the danger of contamination through rupture. Certain hospitals are now using a special grade of parchment paper (27-2T) as a substitute for muslin. This material permits fairly rapid steam transfer and it does not tear easily. Other institutions use small paper bags, usually a white sulphite

bond, for packaging syringes, cotton pledgets, small dressings, etc. for floor use with apparent success and economy.

*Drum Sterilization.*—Experience has demonstrated that there is no good excuse for the use of drums in preparing surgical supplies for sterilization. Even the most modern drums which have more and better distributed portholes in the sides than the older ones seriously retard intake of steam to the contents, especially if the



Fig. 33.—If drums are used, the load must be carefully planned to avoid filling in the open spaces around the walls of the drum. (Courtesy of American Sterilizer Company.)

drum is fully loaded. For those who insist on the use of drums special care should be taken to limit the size and density of the load. Under no condition is it safely permissible to allow the contents to be crowded against the inside wall of the drum. Figure 33 illustrates the proper method of loading. Place the flat packages of sheets, table covers, towels, etc. in the drum but do not fill completely. Do not fill in the open spaces at the sides. When the cover is closed, it must not compress the goods. The contents are surrounded by two thicknesses of muslin covering. Loaded in this manner, sterilization will occur in 30 minutes of exposure. However, if any degree of tight packing is permitted, filling in the side spaces, the exposure period will need to be increased to 45 minutes or longer.



*Rubber Gloves.*—After an operation, the gloves should be washed in cold water before removing them. This detail is often neglected, adding to the difficulties involved in subsequent washing. The gloves are then collected in a suitable container and washed in warm water containing a mild detergent. The use of a scrub brush is recommended in order to loosen adhering particles of dried blood. In many hospitals, an approved type of automatic (laundry) washer is used for this purpose.



Fig. 34.—In wrapping gloves for sterilization, it is good practice to insert a band of gauze or muslin in the wrist fold as shown. Also, a pad of gauze inserted in the palm of the glove will hold the apposing surfaces apart. (Courtesy of American Sterilizer Company.)

Contaminated gloves used in connection with “dirty” cases should be placed in a deep basin or container to which a cold solution of 2 per cent tri-sodium phosphate is added. The container should then be sterilized at  $121^{\circ}\text{C}$  for 45 minutes. Contaminated gloves can also be rendered safe for handling by processing in the modern pressure instrument washer-sterilizer.

Following the washing process, the gloves are tested for leaks and dried. Those found to be punctured are separated from the others and after mending they are not returned to the surgery but are used elsewhere in the hospital for less exacting purposes. The

gloves are then powdered in a confined space such as a glove powdering cabinet or in an automatic glove powdering and drying apparatus. Talcum powder is no longer recommended for this application because of its demonstrated irritating properties which may result in tissue reaction. The product known as "Biosorb," a mixture of

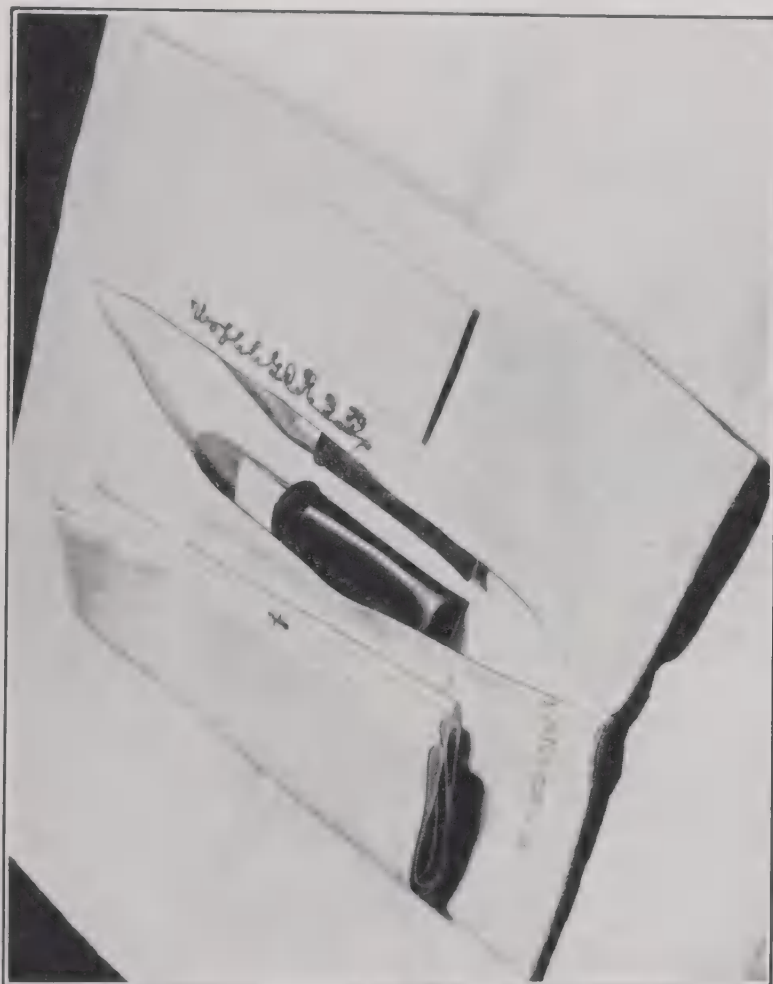


Fig. 35.—The billfold type cover is commonly used for wrapping gloves.  
(Courtesy of American Sterilizer Company.)

mylase and amylase pectinate, has largely replaced talcum powder as a medium for powdering gloves.

In order to completely sterilize gloves, all surfaces must be contacted by the steam. Heat conducted through the walls of a glove, without the moisture factor, is inadequate to destroy even the less resistant pathogens. The usual good grade of rubber will not withstand sterilization for more than 15 to 20 minutes without serious deterioration, nor will rubber stand up in steam tempera-

ture even for short periods of exposure much higher than 121° C. Sterilization must, therefore, be accomplished in 15 to 20 minutes at 121° C. This can be done if the gloves are prepared with care.

First consideration must be given to sterilization of the outer walls, those surfaces exposed on the surgeon's hands during the operation. To this end there must be no crowding, folding or tight wrapping, so that all surfaces are freely exposed to the direct action of the steam. If one surface is in tight contact with another surface, the moisture of the steam may be excluded and sterilization may not occur.

Where the wrist section of the glove is folded back, it is important to keep the two surfaces separated from each other. This can be done by inserting a band of gauze, muslin or paper all the way around inside the fold, as shown in figure 34. As an approach to the ideal, it is also recommended that a pad of gauze or muslin be inserted in the palm of the glove, as far in as the fingers, to hold the apposing surfaces apart and to promote egress of air and intake of steam.

Covers for gloves are usually of the billfold type, with a pocket for one glove on either side of the median line as shown in figure 35. When the gloves are in place, the two sides of the cover are folded together with the openings of each pocket concealed at the center. The pack is held together loosely with tie strings. Some workers prefer to enclose the billfold cover in an outer muslin wrapper for additional protection. Sufficient powder for the surgeon's use can be put up in a small paper envelope or preferably in a gauze sponge. In either form the powder should be included with the gloves for sterilization.

*Rubber Tubing.*—Sterilization of rubber tubing is difficult, yet no possible contamination can be tolerated because of its frequent use in the administering of parenterals or other equally critical applications. Prior to sterilization the tubing plus attachments or connectors should be immersed in a 0.5 per cent solution of sodium hydroxide and boiled for 15 to 20 minutes in order to remove any blood or residual substance remaining in the lumen as the result of previous usage. The tubing should be coiled slowly into the solution to avoid the formation of air pockets, or better yet, the alkali or detergent solution should be circulated slowly through the tubing during the period of boiling.

The tubing should then be attached to a reservoir of freshly distilled (pyrogen-free) water and the water run through it until all traces of alkali or detergent have been removed. Then do not attempt to drain completely or dry the tubing. Rather leave the interior distinctly moist and package immediately by wrapping in



double thickness muslin. The tubing should be carefully coiled but not kinked in the package. The residual moisture plus the heat of the steam conducted to the interior will be sufficient for sterilization when exposed for 30 minutes to a temperature of  $121^{\circ}\text{C}$ .



Fig. 36.—The pressure instrument washer-sterilizer will wash, sterilize and dry two full trays of instruments in a fraction of the time required by the manual process. (Courtesy of American Sterilizer Company.)

*Sterilization of Instruments.*—The selection of a standard method for the pre-operative sterilization of surgical instruments is a serious matter. The sterilization of instruments by boiling is considered by most authorities to be definitely hazardous and to yield little more than sanitization or the destruction of vegetative organisms only. The use of saturated steam under pressure for this purpose affords a greater margin of safety, preserves the life of the instruments, and permits sterilization in a shorter period of time.

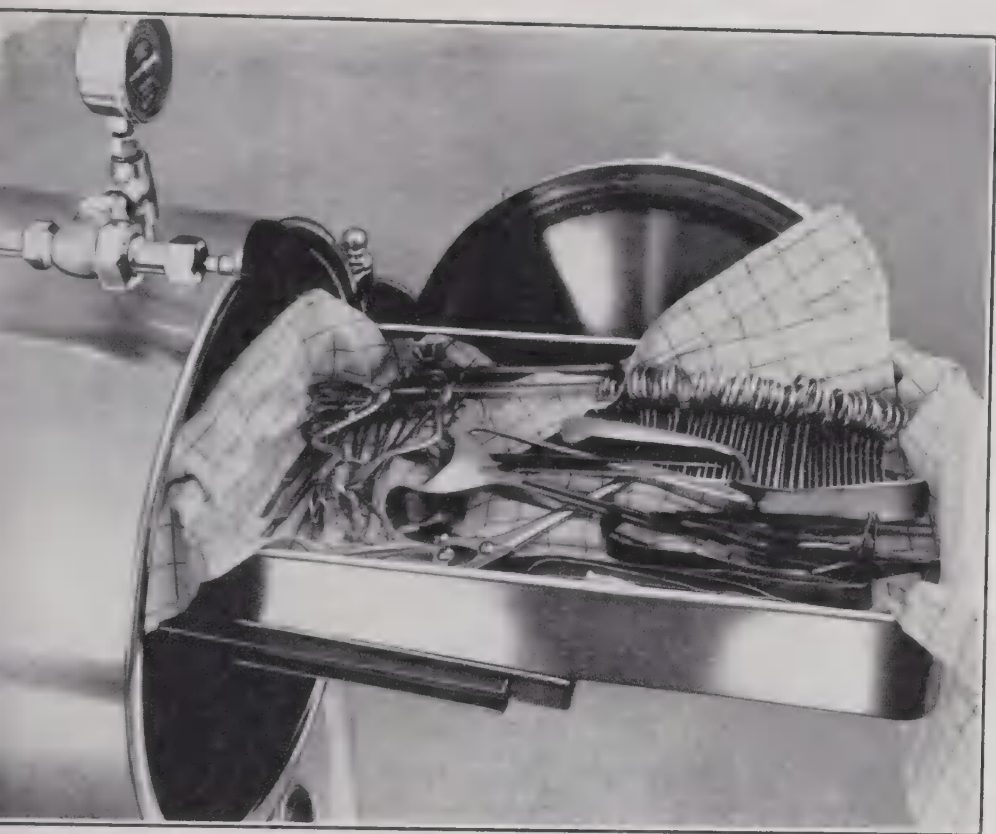
It is essential to clean instruments as promptly as possible after use, to avoid rusting and to remove soil before it can dry and harden in the serrations, crevices, etc. The washing process should be conducted with great care and it should be effective in the removal of all particles of adhering tissue, dried blood, scale or accumulations of lime salts in the serrations, ratchets and box locks of the instruments. A hand brush with fairly stiff bristles may be used to advantage. Also a preliminary soaking of the instruments in water at 52° C (125° F) to which has been added an effective detergent will assist in the removal of inaccessible soil. Thorough cleansing is extremely important because organisms concealed and protected by dried blood and scale in inaccessible parts of the instruments render sterilization more difficult.

Immediately after washing, the instruments should be rinsed with boiling water and then dried thoroughly, while still hot. If any moisture remains on the instruments they may rust in storage. At best, the manual cleaning of instruments is a difficult and time-consuming process. The new mechanical cleansing process, utilizing the pressure instrument washer-sterilizer, (Fig. 36), now widely used in hospitals, makes it possible to employ a superior technique in a fraction of the time normally required for the hand washing of instruments. The oiling of instruments should not be permitted because of the protective action afforded organisms concealed under the film of oil.

For the routine preparation of instruments for sterilization the method of choice is shown in figure 37. The procedure requires placing a towel or layer of muslin in the bottom of the instrument tray. The tray must be provided with a perforated or wire mesh bottom. The instruments are then spread out on the towel, one layer deep, and covered with a second towel or piece of muslin. Make sure that all jointed instruments are open to permit prompt contact of steam to all surfaces. Place heavier instruments above and cover with muslin or another towel for protection against contamination in transit to the operating room. Contact of the instruments with the soft fabric will permit condensate to absorb or vaporize more rapidly and the fabric will also furnish mechanical protection. Sterilize at 121° C for 15 minutes. Following sterilization, the trays of instruments should be allowed to remain in the sterilizer with the door slightly ajar for 10 to 15 minutes to insure thorough drying.

For the emergency sterilization of instruments, the method selected should be adequate for the destruction of resistant spores, and it should be sufficiently rapid so as not to inconvenience the

surgeon. These requirements can be met through the use of the high-speed pressure instrument sterilizer designed for operation at 27 pounds pressure and a temperature of  $132^{\circ}\text{C}$ . At this temperature the exposure period for the emergency sterilization of 1 or 2 instruments is 3 minutes. If a fully loaded and covered tray



g. 37.—Steam under pressure at a temperature of  $121^{\circ}\text{C}$  is the most dependable method for the routine sterilization of instruments. (Courtesy of American Sterilizer Company.)

instruments is to be sterilized at this temperature the exposure period should be extended to 7 minutes.

*Syringes.*—In preparing syringes for sterilization in the autoclave careful attention must be given to thorough cleaning immediately after use and before sterilization, otherwise the sterilizing process may be ineffective, particularly if the syringe contains any clotted or coagulable protein. A syringe that has been used for aspiration, *e.g.*, of blood from a vein, or pus from an abscess, or for intravenous injection, which always entails aspiration of blood, must be cleaned and sterilized before it is used again.



Immediately after use, the plunger should be separated from the barrel and both parts, including needle, rinsed with cold water. This step should be followed by washing in warm water to which has been added an efficient (non-alkaline) detergent. The use of a fiber test-tube brush will facilitate cleaning inside of barrel. After

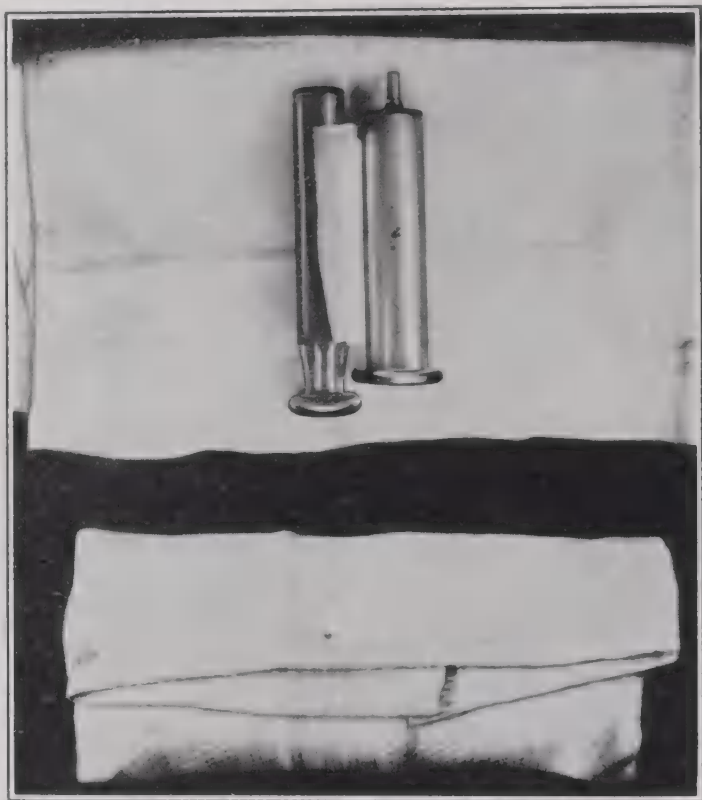


Fig. 38.—A common method of preparing syringes for steam or hot air sterilization. The plunger is removed from the barrel and the two parts wrapped in a muslin cover. (Courtesy of American Sterilizer Company.)

washing, the syringe parts should be rinsed in three changes of distilled water and then permitted to air-dry.

Unless certain precautions are observed in preparing syringes for autoclaving, the incidence of breakage may be greater than when dry heat is used as the sterilizing medium. It is not considered good practice to attempt sterilization of assembled syringes in the autoclave because of the uncertain steam penetration to the sliding surfaces between barrel and plunger. Also, bursting fractures are likely to occur as the result of uneven cooling of the barrel and plunger when steam is exhausted quickly from the sterilizer.

A common method of packing syringes is to wrap the barrel and plunger together in one muslin cover as shown figure 38. If desired, the needle embedded in gauze can be included in the package. With this method of preparation syringes can be sterilized along with bulk loads of heavier supplies at  $121^{\circ}\text{C}$  for 30 minutes.

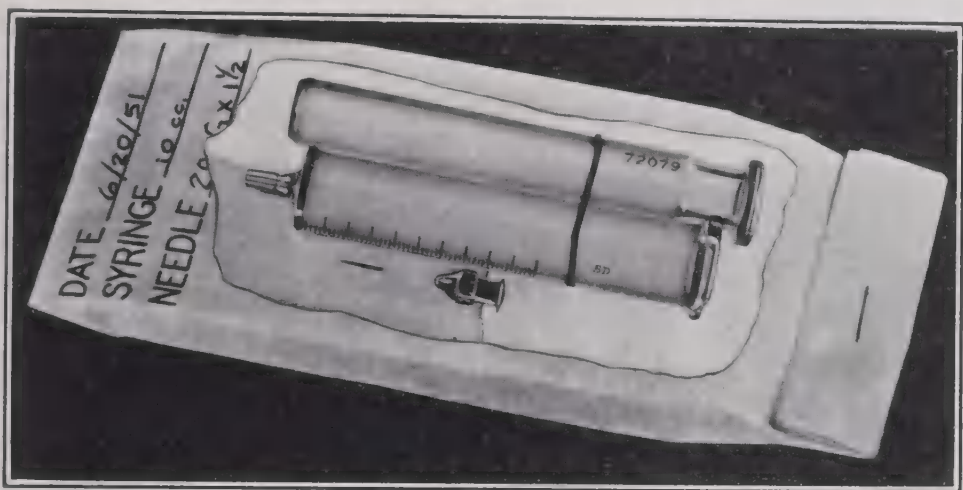


Fig. 39.—A paper bag of the proper size and characteristics offers a convenient means of packaging syringes for sterilization in the autoclave. (Courtesy of American Sterilizer Company.)

A newer technique which lends itself to rapid preparation and sterilization of syringes by the autoclaving process is now widely used in hospitals, laboratories and clinics. This method as illustrated in figure 39 employs a specially fabricated paper bag of high bursting strength and maximum steam permeability characteristics. Syringes prepared in this manner may be sterilized at  $121^{\circ}\text{C}$  for 20 to 30 minutes. When ready for use, the top portion of the bag is torn off, the plunger inserted in the barrel, and the tip of the syringe slipped into the hub of the needle.

*Loading Supplies in the Sterilizer.*—The fundamental rule in loading the sterilizer is to prepare all packs and arrange the load in such a manner as to present the least possible resistance to the passage of steam through the load, from the top of the chamber toward the bottom. Articles such as sheets, table covers, towels, etc., which constitute the difficult-to-permeate-with-steam group must be arranged in the sterilizer so that they rest on edge rather than flat side up, in order to permit prompt and complete permeation with steam. If there are several tiers of flat packs, place alternate tiers crosswise of each other to promote steam circulation.

Figure 40 illustrates an ideal arrangement of loading for the usual surgical supply sterilizer. The load has been laid out on a table top just as it is to be located in the sterilizer. The packs are not tightly wrapped and the upper layer is placed crosswise of the lower layer. All packs are resting on edge, in loose contact with each other. Following sterilization for 30 minutes at  $121^{\circ}\text{C}$ , the normal drying period for such packs should be not less than 15 minutes, with the door of the sterilizer slightly ajar or by means of the vacuum drier process.

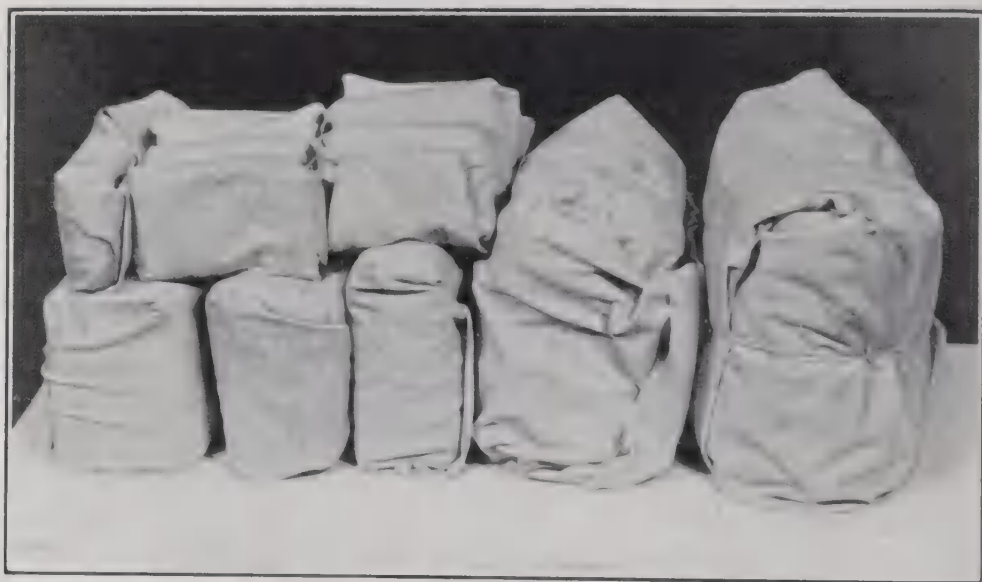


Fig. 40.—Proper arrangement of the load in the sterilizer is essential to successful sterilization. All packs should rest on their sides so that flat article are on edge, to promote rapid permeation of steam through the mass. (Courtesy of American Sterilizer Company.)

If jars (enamelware or metal) or other non-porous containers are used for small dressings, cotton pledgets and the like, care should be taken to insure that the contents are not densely packed. Figure 41 shows the right and wrong way to place such jars in the sterilizer. In the upright position, air is trapped within the jar, even if the cover is removed, and sterilization will be most difficult, if not impossible, in any reasonable period of exposure. If the jar has a loose fitting cover and is placed on its side in the sterilizer, air will drain out and steam will promptly take its place. Such jars or containers usually have covers that can be secured in place *loosely* by tying the covers on with tape, so they cannot fall off when placed in the sterilizer on their sides. The period of exposure should be 30 minutes at  $121^{\circ}\text{C}$ .



An excellent rule to follow in sterilizing any dry material contained in a test tube or jar is to assume that the container is filled with water, then place it in the sterilizer in such a position that the water will drain out freely. Similarly, in the presence of steam, air will drain out freely and steam will then enter the container unretarded.

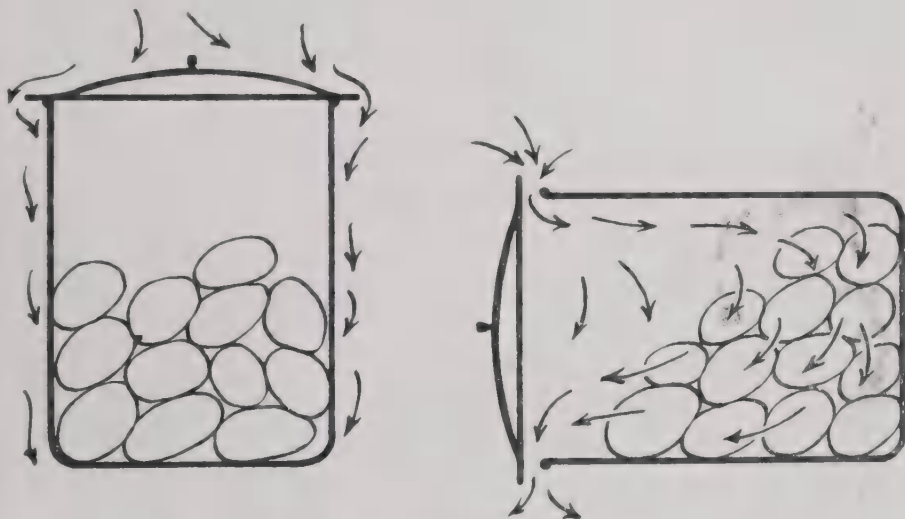


Fig. 41.—When placing jars of dressings in the sterilizer care should be taken to insure that they rest on their sides in order to provide a horizontal path for the escape of air. When right side up, even with the cover removed, air is trapped in the container. (Courtesy of American Sterilizer Company.)

Glove packs should also rest on edge in the sterilizer, well away from the side walls of the chamber and in contact with no other part of the load. They must never be crowded together into a tight mass and preferably they should be sterilized by themselves. The instrument sterilizer tray with wire-mesh bottom (Fig. 42) makes an excellent container for gloves. It is imperative that the gloves be stacked loosely, never more than one tier deep.

*Sterilization of Liquids.*—Sterilization of liquids in test tubes, flasks or bottles involves a different use of steam than is required for dry goods sterilization. In solution sterilization the problem is simply a matter of absorbing heat from the steam. The solution (aqueous) contains the necessary moisture. The time required for heating governs the exposure period and this depends mainly on the rate of heat transfer through the walls of the container and the ratio of exposed heat-absorbing wall surface to the volume of liquid. Measurements to determine the time required for a given container to attain a temperature of  $121^{\circ}\text{C}$  are usually made by

means of a potentiometer and thermocouple. The graphic data given in figure 43 are typical of the different rates of heat transfer through commonly used flasks and bottles.

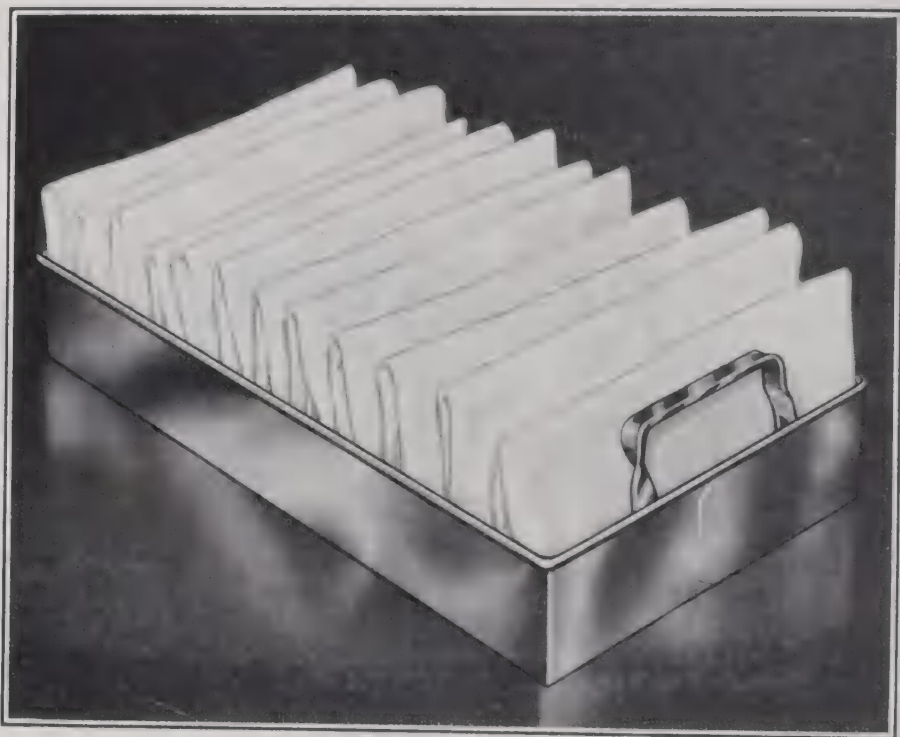


Fig. 42.—The instrument sterilizer tray with wire-mesh bottom makes an ideal container for gloves. The packs should rest on edge in the sterilizer. (Courtesy of American Sterilizer Company.)

The cycle of operation in solution sterilization should be understood in order to avoid common errors. While the solution is being heated and as long as exposure continues there will be no ebullition of the liquid, even though the temperature is far higher than the normal (atmospheric) boiling point of water. This is due to the steam pressure in the chamber, at all times equal to or in excess of the pressure possible to develop from the heat of the liquid. When the chamber pressure is reduced, after sterilization, the condition reverses, and unless the pressure is exhausted slowly the solution will boil violently, stoppers will be blown out of the flasks and some of the solution will escape into the chamber.

When the pressure is exhausted at a uniform rate through a period of not less than 10 to 12 minutes, the solution will lose its heat at about the same rate, so that violent boiling will not occur. Even when the cooling down process is conducted carefully there will be an evaporation loss of fluid of 3 to 5 per cent. For this reason

it is good practice in preparing solutions to add about 5 per cent additional distilled water so that the sterilized product will have the desired concentration. Solutions may also be cooled down by

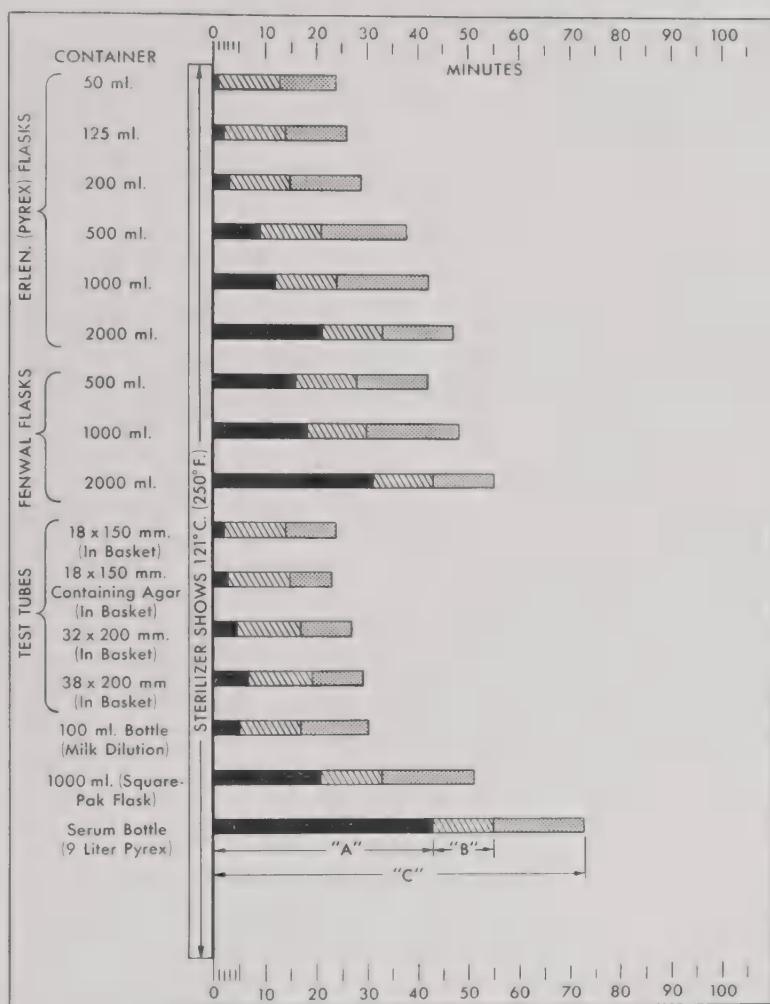


Fig. 43.—Comparison of the different rates of heating of solutions in commonly used containers. Section "A" of each bar represents the additional time required for the solution in the container to reach 121° C after the sterilizer thermometer shows this temperature. Section "B" is the holding period (minimum standard) after the solution has reached 121° C. "A" plus "B" equals the required exposure period. The entire bar length designated "C" is descriptive of the total time that the solution remains above 100° C during the entire sterilizing cycle, including slow exhaust. (Sterilizer equipped with automatic control.)

turning off all heat to the sterilizer and permitting it to cool slowly to atmospheric pressure before opening the door. This procedure is satisfactory for most solutions but it requires a considerable period of time. Heat-sensitive solutions will usually be affected by this prolonged exposure to temperatures above 100° C.



In a modern sterilizer, operated in accordance with the manufacturer's instructions, the following exposures for aqueous solutions will afford a reasonable degree of safety in sterilization:

<i>Container</i>	<i>Capacity</i>	<i>Minutes Exposure</i> <i>121-123°C</i>
Erlenmeyer (Pyrex) Flask		
"          "          "	2000 ml	30-35
"          "          "	1000 ml	20-25
"          "          "	500 ml	17-22
"          "          "	200 ml	12-15
"          "          "	125 ml	12-14
"          "          "	50 ml	12-14
Fenwal (Pyrex) Flask	2000 ml	40-45
"          "          "	1000 ml	25-30
"          "          "	500 ml	24-28
"Square-Pak" (Pyrex) Flask	1000 ml	30-35
Serum Bottle (Pyrex)	9000 ml	50-55
Milk Dilution Bottle	100 ml	13-17
Test Tubes	18×150 mm	12-14
"          "	32×200 mm	13-17
"          "	38×200 mm	15-20

*Bacteriological Media.*—Various kinds of bacteriological media are recognized to be heat-sensitive. Carbohydrates, in particular, when present in media frequently hydrolyze during heat sterilization. Phenol red lactose broth, for example, sterilized for 15 minutes at 121° C, or sterilized by filtration, produces no demonstrable amount of acid when inoculated with *S. typhosa*, but when sterilized for 30 to 45 minutes at this temperature an appreciable amount of acid is produced.

It has also been observed that prolonged sterilization may result in the formation of a precipitate in agar media. Such over-exposure of the media may also cause an increase in acidity as the heating is prolonged. Media, such as wort agar with a normal pH of 4.8, will upon over-sterilization, cause destruction of the agar. It is possible to destroy completely the jellifying properties of agar and gelatin by excessive heating. This destruction is hastened by the increase in acidity. The skillful bacteriologist distributes and sterilizes nutrient agar immediately after it has been made and while it is still fluid because of the destructive influence attendant with each additional heating. A common error is to sterilize small amounts, such as 10 ml in 18x150 mm test tubes, of heat-sensitive fluids at the same time as fluids in larger containers are sterilized. Either the fluid in the tubes will be heated much longer than is necessary or the fluid in the large containers will escape sterilization.

Certain types of media such as blood serum slants, Loewenstein's, Petroff's and other heat-coagulable media call for special treatment in order to avoid formation of bubbles caused by engendered pressure. These media require processing temperatures ranging from 100° C down to 80° C. Whereas in the past it has been necessary to process heat-coagulable media in the inspissator or Arnold flowing-steam sterilizer, recent developments have made it possible to extend the applications of the modern laboratory autoclave so as to permit this type of performance.

## DRY HEAT STERILIZATION

The subject of dry heat sterilization, more commonly referred to as hot air sterilization, is often poorly understood by those responsible for sterilizing techniques in the average hospital or laboratory. Perhaps this lack of understanding is due to the primary emphasis placed on the use of saturated steam under pressure as the most dependable medium known for the destruction of all forms of microorganisms. That this statement is true cannot be denied, but it is also important to note that pressure steam has certain limitations which make it unsuitable for the sterilization of materials such as anhydrous oils, greases, powders, etc. Because of their physical characteristics these materials cannot be permeated by the moisture of the steam and sterilization must, therefore, be accomplished by means of dry heat in a hot air oven.

Occasionally operators assume that inasmuch as a substance is normally fluid or becomes fluid under heat, as in the case of petroleum jelly, the objective of sterility can be attained by subjecting the substance to the normal autoclaving process. This is a hazardous assumption due to the fact that petrolatum, oils, oily suspensions, fats and powders have no appreciable water content, the moisture of the steam cannot permeate the substances, and, as the result, sterilization cannot be accomplished within a reasonable exposure period regardless of how efficient the autoclave may be. Heat resistant organisms concealed in such products will be heated to the temperature of the surrounding steam, but lacking the moisture factor, the temperature will be inadequate for complete bacterial destruction.

*Comparison of Steam and Dry Heat in Sterilizing Processes.*—In comparing the effects of moist heat (steam) with dry heat in sterilizing processes, it is necessary to give consideration to the mechanism responsible for the destruction of bacterial life. In the case of moist heat it is generally conceded that death occurs as the result of coagulation of protein within the bacterial cell. Certainly

it is known that water is necessary for coagulation of proteins, and destruction of bacteria by moist heat is probably due to this process.

Various workers have attempted to explain the phenomena responsible for the widely different temperatures required in dry and moist heat sterilization on the basis of the changes in the coagulability of proteins brought about by the abstraction of water. Lewith (1890) found that various proteins are coagulated by heat at lower temperatures when they contain an abundance of water, than when water has been abstracted from them. The following data illustrate the point in question:

Egg Albumin + 50% Water	Coagulates at 56° C (133° F)
Egg Albumin + 25% Water	Coagulates at 74°-80° C (165°-176° F)
Egg Albumin + 18% Water	Coagulates at 80°-90° C (176°-194° F)
Egg Albumin + 6% Water	Coagulates at 145° C (293° F)
Egg Albumin + 0% Water	Coagulates at 160°-170° C (320°-338° F)

The above data indicate that a temperature of at least 160° C (320° F) should be used in dry heat methods of sterilization. Bacteria exposed to hot air may be dehydrated greatly before the temperature rises sufficiently to cause death by coagulation. For this reason, most authorities concur that death by dry heat is primarily an oxidation process.

When compared with steam, the limitations of dry heat as a sterilizing agent should be clearly understood. It is recommended only where direct contact of the material or substance with pressure steam is impractical or unattainable. Dry heat in the form of hot air is difficult to control within narrow limits. It penetrates materials slowly and unevenly and long exposure periods are required for sterilization. Because of the poor penetrability and the destructive effect of the high temperatures employed, dry heat or hot air is entirely unsuited for the sterilization of fabrics and rubber goods.

*Resistance of Bacteria to Dry Heat.*—The unusual resistance of bacteria, particularly spores, to dry heat temperatures has long been recognized. The early findings of Robert Koch (1881) and his confreres clearly demonstrated that the spores of *Bacillus anthracis* required a hot air temperature of 140° C for 3 hours in order to insure their destruction. A review of the literature has shown that there is a lack of systematic study on death time temperatures of dry heat (hot air) as compared to moist heat or steam. From the available data it is reasonable to conclude that an exposure to dry heat at 160° C for 60 minutes is approximately the equivalent of an exposure to moist heat at 121° C for 10 to 15 minutes.

The resistance of both vegetative bacteria and spores varies considerably with different species, some being killed more rapidly



han others. The spores of molds appear to be intermediate in resistance between vegetative and sporulating bacteria in that they require a temperature of  $110^{\circ}$  to  $115^{\circ}$  C for 90 minutes for their destruction. The data summarized in Table 113 are descriptive of the findings of various investigators in determining the time-temperature ratios required for destruction of bacterial spores by means of dry heat.

It is of special importance to note that the microbicidal action of dry heat is markedly influenced by the nature of the fluid or substances surrounding the organism. In the presence of organic matter such as a film of oil or grease the organism is definitely protected or insulated against the action of dry heat. Walter (1948) has emphasized the importance of this factor in dry heat sterilization, particularly in the case of surgical instruments, which, if properly cleaned beforehand, may be sterilized in one hour at  $160^{\circ}$  C. If oil or grease is present on the instrument, safe sterilization calls for 4 hours' exposure at  $160^{\circ}$  C.

The thermal death time-temperatures of resistant dry spores in anhydrous oil have been carefully studied by Rodenbeck (1932). The findings of this investigator are deserving of serious consideration in the establishment of safe exposure periods for dry heat sterilization of oils, fats or other anhydrous fluids. For example, it has been determined that at a temperature of  $160^{\circ}$  C a period of 60 minutes is required for destruction of resistant spores in anhydrous oil or fat. If the oil is hydrated or contains a small amount of water, as little as 0.5 per cent, sterilization may be accomplished in approximately 20 minutes at this temperature, but the moisture must be retained during sterilization. Most oils contain a small amount of water (less than 1 per cent) unless subjected to a specific dehydration process.

*Minimum Requirements for Dry Heat Sterilization.*—Due to the various factors involved in dry heat sterilization, it is difficult and somewhat impractical to attempt to establish one time and temperature entirely suitable for all types of loads. Not only must the characteristics of the material undergoing sterilization be known, but strict attention must also be given to the method of preparation, packaging or wrapping, and loading of the sterilizer to insure that the exposure period selected will be adequate for the destruction of the most resistant and least accessible organisms.

For certain materials, such as glassware, it becomes possible to employ a higher temperature for a shorter period of time than when sterilizing powders which may undergo physical or chemical change unless the temperature is maintained below the critical point of the substance. Instruments represent the ideal for dry heat

TABLE 113. — DESTRUCTION OF BACTERIAL SPORES BY DRY HEAT AT DIFFERENT TEMPERATURES  
(DATA FROM PERKINS, 1951)

Organism (dry spores)	Time—minutes							Investigator
	120° C (248° F)	130° C (266° F)	140° C (284° F)	150° C (302° F)	160° C (320° F)	170° C (338° F)	180° C (356° F)	
B. anthracis	45	20						Murray (1931)
B. anthracis			180					Koch <i>et al.</i> (1881)
B. anthracis				60				Stein and Rogers (1945)
B. anthracis			180					Park and Williams (1933)
B. anthracis	60							Oag (1940)
B. subtilis				60	9			Perkins and Underwood (1945)
Cl. botulinum	120	60	60	25	25	15	10	Tanner and Dack (1922)
Cl. septicum						7		Oag (1940)
Cl. tetani		35	15					Murray and Headlee (1931)
Cl. welchii		15	5					Headlee (1931)
Cl. welchii	50					7		Oag (1940)
Garden soil					30	15		Ecker and Smith (1937)

sterilization because of the heat conducting properties of the metal, but here again the maximum temperature employed for sterilization must be restricted to a safe range beyond which the temper may be drawn. Everyone concerned with sterilizing techniques should constantly keep in mind that the time required to heat a quantity of one material to sterilizing temperature may differ markedly from that required to heat another material to the same temperature.

The most widely used temperature for dry heat sterilization of hospital supplies is 160° C for a period of not less than 1 hour, preferably 2 hours. This requirement refers to the actual temperature of the load and does not compensate for any appreciable time lag characteristic of a particular load after the sterilizer has reached this temperature. In establishing reliable methods for dry heat (hot air) sterilization, the following time-temperature ratios are recommended:

170° C (340° F)	60 minutes
160° C (320° F)	120 minutes
150° C (300° F)	150 minutes
140° C (285° F)	180 minutes
121° C (250° F)	Overnight

*Electrically Heated Hot Air Sterilizers.*—The electrically heated hot air sterilizer is preferred to other types because of its facility for accurate and reliable temperature control within the desired range. For routine work this temperature range should be 160° to 170° C, but the automatic regulator should be readily adjustable for lower temperature sterilization as in the case of heat-sensitive materials such as zinc peroxide.

When hot air sterilization is used in the laboratory for glassware only, close control of temperature is not so essential and is rarely practiced. The temperature may advance to as much as 200° C without injury to the glassware. For this reason the laboratory hot air sterilizer is frequently just a plain, uninsulated, gas-fired oven, without thermostatic control. Gas cannot be controlled with the same accuracy as electricity and the use of gas involves a definite fire hazard. Such equipment has no place in the modern laboratory and it should not be used for the sterilization of materials that require accurate temperature control or are known to be combustible in nature.

*The Gravity Convection Type.*—There are two distinct types of electrically heated hot air sterilizers. The gravity convection model is commonly used in laboratories. In this unit, air circulates in accord with existing temperature differences between various



portions of the chamber, as illustrated schematically in figure 44. When air is heated, it expands with a corresponding decrease in density. The colder air descends in the chamber and the heated air rises to displace it. The ascending warm air gives up some of its heat to the load in the chamber and also contracts in volume. At the same time the descending cool air is heated as it passes over the heating elements. Thus the cycle is repeated, setting up "gravity convection" circulation in the chamber. The speed of circulation

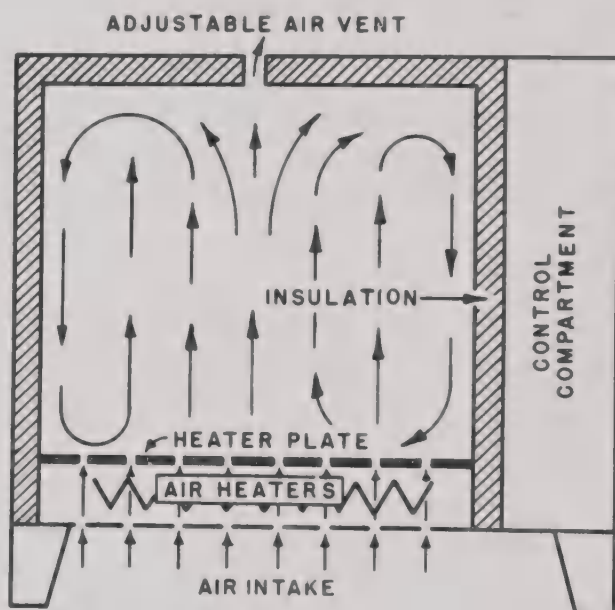


Fig. 44.—Schematic diagram of air circulation in gravity convection hot air sterilizer. (Courtesy of American Sterilizer Company.)

is dependent upon the ventilation afforded through the adjustable air vent or exhaust located on top of the sterilizer and the temperature differential between the region of the heat source and the exhaust port.

In selecting a gravity convection hot air sterilizer, care should be taken to insure that the design does not oppose the natural flow of air currents by directing the air stream around corners, through baffles, etc. The heater bank should be located beneath the chamber, separated from it by a perforated metal plate, which serves not only as the chamber floor, but as a diffusing surface to produce a uniform heating effect over the full horizontal plane of the working chamber. Pre-heated fresh air can then rise through the perforated plate, pass up through the chamber, flow through the perforated diffusing panel which forms the ceiling, and finally exhaust itself through the adjustable ventilating port on top of the cabinet.

The gravity convection type sterilizer is much slower in heating, requires longer time to reach sterilizing temperature, and possesses less uniformity of temperature control throughout the chamber than is evident in the mechanical convection model. The former is suitable only for applications where rapid and precise heating, limit of chamber loadability, and accelerated air circulation are not decisive factors.

*The Mechanical Convection Type.*—For the normal requirement, whether it be in the laboratory, surgery or central sterile supply department, the mechanical convection hot air sterilizer is the preferred type. The advantages inherent in this particular design are worthy of emphasis. By referring to the schematic diagram (Fig. 45), it will be observed that the sterilizer is equipped with a motor-driven turbo-blower which produces a rapid movement of a large volume of heated air, to convey heat directly to the load under controlled temperature conditions. The heater bank is mounted in a compartment separated from the working chamber by a diffusing wall, directly in front of the turbo-blower. An adjustable air inlet opens to the heater compartment. Incoming air is heated, then enters the turbo-blower where it is thoroughly mixed and diffused with recirculating air.

From the turbo-blower, the heated air passes through a duct, where a high static pressure is built up, forcing the air over to a compartment on the opposite side of the chamber. Here it passes through another perforated diffuser wall and the air is discharged uniformly over the entire vertical plane area of the chamber. This ensures a positive airflow in a horizontal plane, thus maintaining uniform temperature and equal transfer of heat to all regions. As the heated air flows across the chamber and passes through the diffusing wall in front of the heaters, it is then recirculated by the turbo-blower and the cycle repeated. Any portion of the circulating air can be exhausted to the atmosphere through the adjustable vent shutter located on top of the sterilizer.

With the mechanical convection sterilizer, air velocity, air pressure, direction of circulation and heat intensity are definitely controlled—to produce uniform temperature in the chamber regardless of its contents. The features of increased loading capacity, positive exhaust of gases or vapors liberated during the sterilizing process and speed of operation constitute additional advantages not obtainable with the gravity convection type hot air sterilizer. Mechanical convection reduces operating costs because it saves on current consumption while performing a large volume of work in a minimum of time.

*Use of Autoclave as Hot Air Sterilizer.*—Operating room supervisors and others responsible for sterilizing techniques in hospitals and laboratories frequently attempt to use the pressure

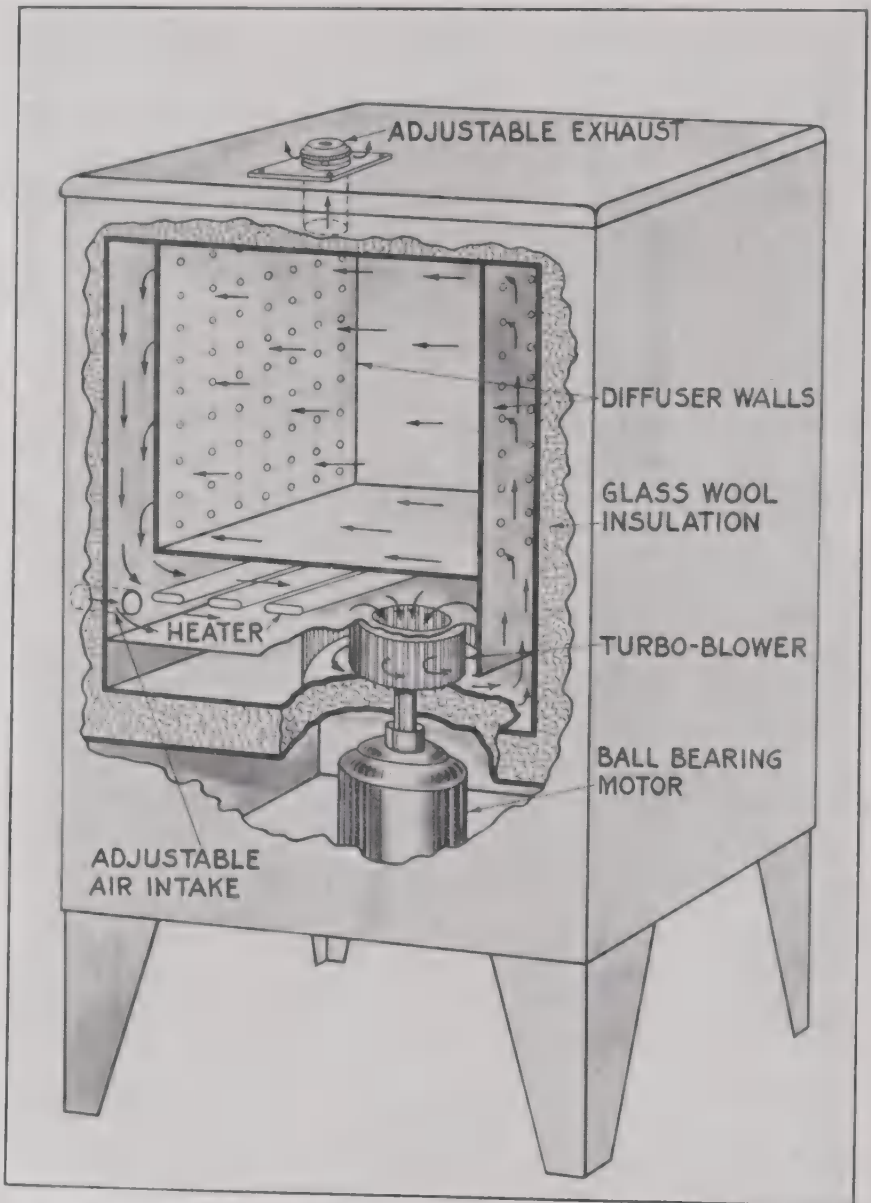


Fig. 45.—Schematic diagram of air circulation in mechanical convection hot air sterilizer. Heat transfer by mechanical convection offers high efficiency for all sterilizing applications. (Courtesy of American Sterilizer Company.)

steam sterilizer (autoclave) with steam in jacket only as a substitute for the hot air oven. This can be done but the method is definitely not satisfactory as a standard procedure. The commonly



used dressing sterilizer is not designed or constructed to serve efficiently as a dry heat sterilizer.

When operated with steam in the jacket only at 15 to 17 pounds pressure, it is true that the chamber walls of the sterilizer are uniformly heated to  $121^{\circ}\text{C}$ , and that conditions in the chamber are moderately suited to hot air sterilization. However, it should be noted that the thermometer located in the chamber discharge line does not function as an indicator of the chamber temperature when steam is applied to the jacket only. This introduces a serious objection to the method because there is no reliable means of checking temperature conditions in the chamber, without modification of the sterilizer.

Another objection to the use of the autoclave as a substitute for the hot air sterilizer resides in the fact that the standard steam pressure in the jacket of the dressing sterilizer is 15 to 17 pounds, equivalent to  $121^{\circ}$  to  $123^{\circ}\text{C}$ . With this (dry heat) temperature in the chamber, reliable sterilization calls for an exposure of overnight. Such a long period of exposure is indicative of a relatively inefficient process with a lesser margin of safety than is achieved through the use of a standard hot air sterilizer operated at a higher temperature for a shorter period of time. It is also true that the long (overnight) exposure period almost prohibits the use of the autoclave for this purpose during the day. As the result, the busy hospital may be severely handicapped in production capacity of those supplies requiring hot air sterilization.

In the case of oils, greases and lubricated gauze, it is not uncommon to experience spillage or leakage of these materials in the sterilizer chamber. When this occurs in the autoclave, it necessitates a careful cleaning operation to remove all traces of oil or grease from the chamber surfaces, otherwise supplies undergoing pressure steam sterilization may later become contaminated with a film of oil which serves as a protective barrier against moist heat sterilization. Furthermore, it is well known that oil or grease in the chamber eventually leads to partial clogging of the discharge line which requires careful cleaning and service by an experienced mechanic to insure efficient operation of the sterilizer. The obvious answer to all of these shortcomings is not to use the autoclave as a substitute for the hot air sterilizer, except in an emergency or until such time as suitable equipment designed and constructed for this application can be procured.

## PREPARATION OF SUPPLIES

Careful attention to details in the preparation of supplies for dry heat sterilization cannot be over-emphasized. Wherever prac-

ticable the quantity of the material should be limited to that required for a single application. An attempt should be made to standardize on the type of container, the method of packaging or wrapping and loading to insure that when the sterilizer reaches the proper temperature the load also will be at this temperature or very close to it.

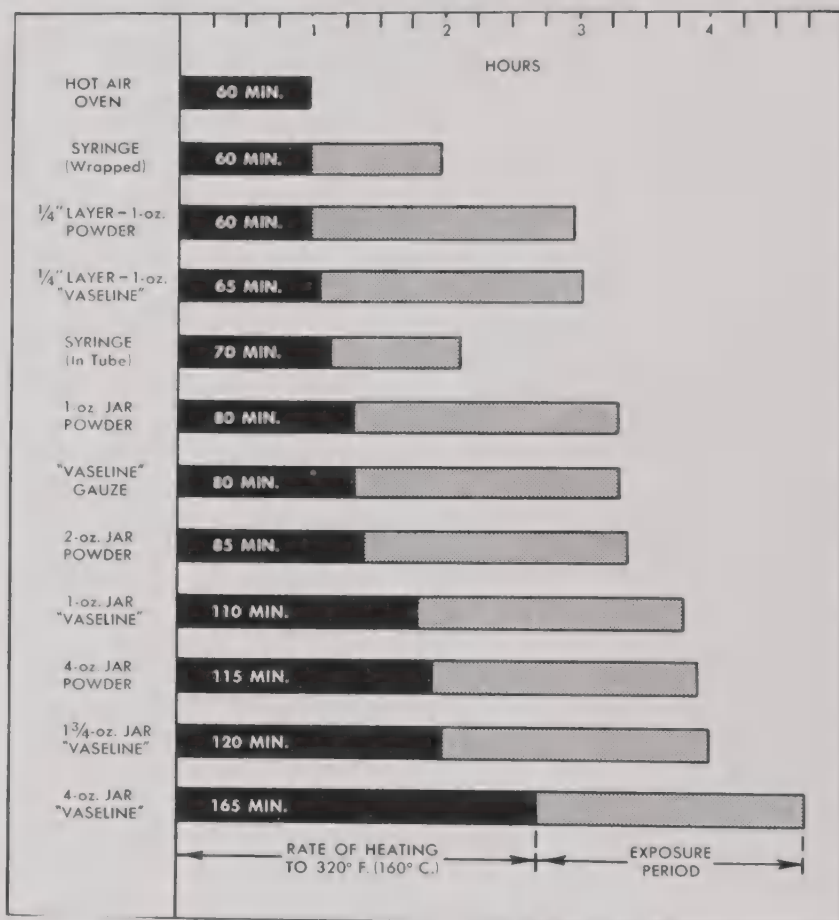


Fig. 46.—Time required to heat specific quantities of materials from room temperature of 25° to 160° C in hot air sterilizer. The lightly shaded section of each bar is descriptive of the exposure period after the material has attained a temperature of 160° C. The total time consumed in the heating process is equivalent to the entire length of each bar. (Courtesy of American Sterilizer Company.)

*Powders.*—In almost any type of jar or container filled with powder the rate of heat transfer is so slow that it becomes necessary to employ abnormally long periods of heating to insure sterilization. This point is illustrated graphically in figure 46. Here it will be observed that a 4 oz jar of powder of the type shown in figure 47 required 115 minutes to reach the sterilizing temperature of 160° C in the hot air sterilizer. From the time that the sterilizer

itself had reached this temperature, the powder still required 55 minutes longer. Then, for sterilization it is necessary to add an exposure period of 2 hours which would make the total heating time about 4 hours.

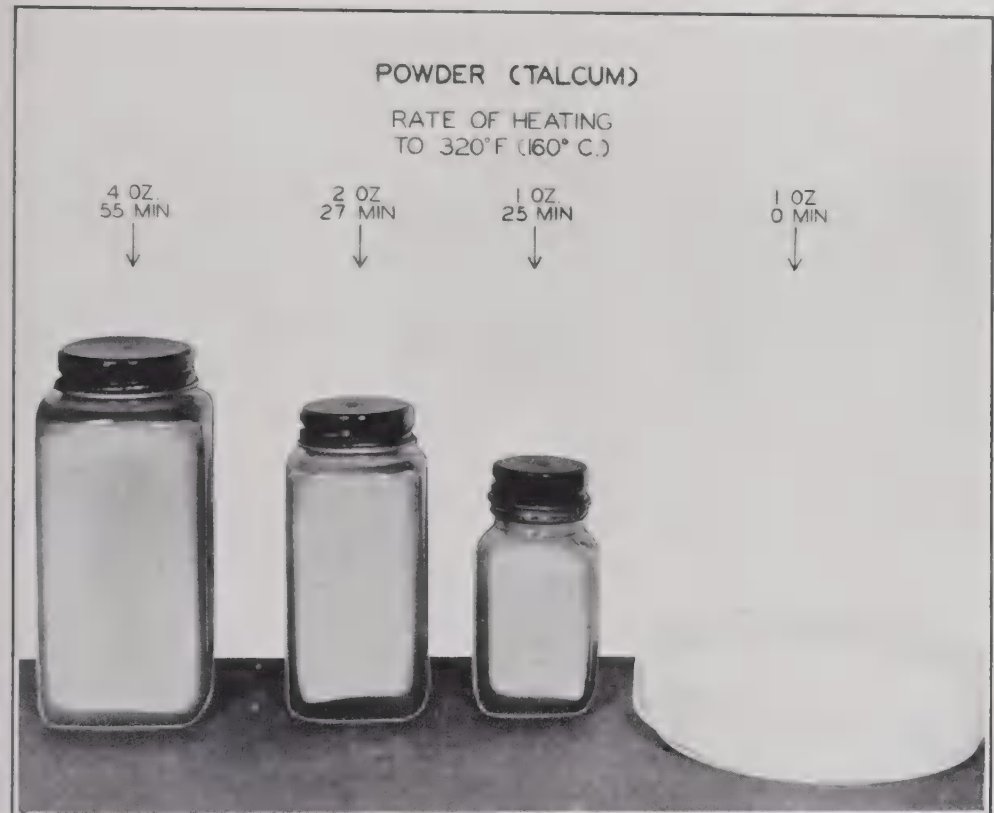


Fig. 47.—Comparative rates of heating of powder in various quantities and containers to 160° C (320° F) in hot air sterilizer. The time recorded for each container is actually the additional time needed for the powder to reach 160° C after the sterilizer has attained this temperature. (Courtesy of American Sterilizer Company.)

Further reference to figure 46 shows that similar conditions of slow heating prevail for the 2 and 1 oz jars of powder. The influence of the depth of the layer and the surface area exposed provide a logical explanation of the slow heating factor of powder. A  $\frac{1}{4}$ -inch layer, amounting to 1 oz of powder, was placed in a Petri dish as shown in figure 47 and then heated in the hot air sterilizer. By means of a potentiometer and thermocouple, it was determined that the temperature rise, after 30 minutes, ran parallel to that of the sterilizer, so that after 60 minutes of heating both were at the same temperature of 160° C. This is typical of the ideal condition—uncommonly practiced in the hot air sterilization of powders.



With the above as a background, the author is compelled to stress the importance of discouraging the use of containers for bulk powder involving quantities greater than 1 oz. Wherever possible the quantity of powder should be limited to a  $\frac{1}{4}$ -inch layer in a Petri dish or a similar type of container. Otherwise the exposure period of 2 hours at  $160^{\circ}\text{C}$  may be inadequate.

*Sulfonamide Powders.*—Sulfanilamide, sulfapyridine and sulfathiazole in powder form may be heated in small amounts in the hot air oven to a temperature of  $155^{\circ}\text{C}$  for  $1\frac{1}{2}$  hours without decomposition. However, this temperature is very close to the melting point of sulfanilamide especially ( $165^{\circ}\text{C}$ ), and for a greater margin of safety as far as thermostability is concerned, it is recommended that the powders be maintained at  $140^{\circ}$  to  $150^{\circ}\text{C}$  for not less than 3 hours.

A convenient method of preparation is to place small amounts of the powder, not exceeding 4 or 5 g, in double envelope paper containers. Test tubes with cotton plugs, individually wrapped, also serve as suitable containers.

*Zinc Peroxide (Medicinal Grade).*—In preparing this material for clinical use, 15 to 20 g lots in suitable containers or test tubes have been found satisfactory. The heating must be carried out in an oven at  $140^{\circ}\text{C}$  to effect sterilization and also to mobilize the oxygen. To be effective this material must conform to certain minimum standards which have been outlined in detail by Meleney (1948). The reader should consult this reference for further information.

*Various Oils (Petrolatum, Petroleum Jelly, etc.).*—The slow rate of heating evidenced in the sterilization of powders is even more marked in the case of oils. For example, the time required to heat a 4-oz jar of petroleum jelly from room temperature of  $24^{\circ}\text{C}$  to  $160^{\circ}\text{C}$  in the hot air oven amounted to 165 minutes, as shown in figure 46. From the time that the oven had reached this temperature, the petroleum jelly required 100 minutes longer heating to reach  $160^{\circ}\text{C}$ . If the exposure period of 2 hours is then added, it is apparent that the total heating time is about  $4\frac{3}{4}$  hours for sterilization of this quantity of petroleum jelly.

The time required to heat smaller quantities of petroleum jelly to sterilizing temperature is far greater than is commonly known and observed in hospital practice. The data recorded in figure 46 show that a  $1\frac{3}{4}$ -oz jar requires 120 minutes and a 1-oz jar 110 minutes to  $160^{\circ}\text{C}$ . This means that when the sterilizer shows  $160^{\circ}\text{C}$  an additional period of 60 minutes and 50 minutes respectively is required for the two smaller containers to reach sterilizing temperature.

As in the case of powder explained above, the depth of the layer of oil and the surface area exposed greatly influence the rate of heating. To illustrate, a  $\frac{1}{4}$ -inch layer of petroleum jelly,  $1\frac{3}{4}$  oz., was placed in a Petri dish, as shown in figure 48 and then heated in the hot air sterilizer. The temperature rise, as determined by means of a potentiometer and thermocouple, ran almost parallel to that of the sterilizer after 30 minutes of heating, so that at the end of 60 minutes the petroleum jelly required only 3 minutes longer

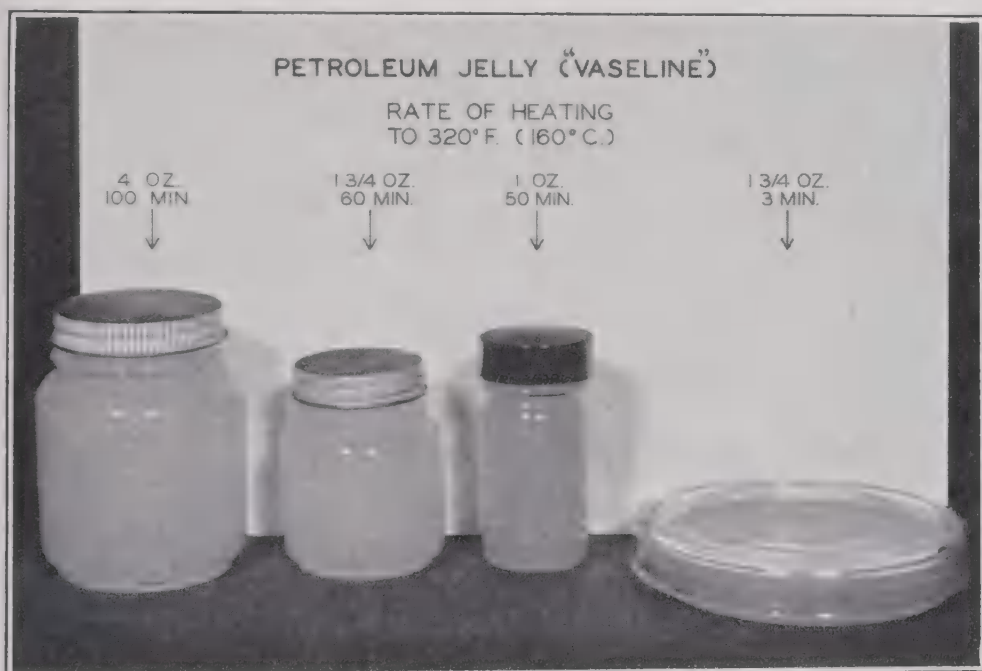


Fig. 48.—Comparative rates of heating of petroleum jelly in various quantities and containers to 160° C in hot air sterilizer. (Courtesy of American Sterilizer Company.)

heating to reach 160° C. Again, this condition approaches the ideal, and the results confirm the recommendation that the quantity of oil should be limited to about the amount needed for a single application.

Although the Petri dish is not the most suitable type of container for oils, because of its shallow depth and the possibility of spillage, it can serve as a convenient receptacle for petroleum jelly, ointments or other materials not normally liquid at room temperature. Certain hospitals prefer to use a stainless steel needle jar,  $3\frac{1}{8} \times 2\frac{1}{2}$  inches, with cover, as a container for petroleum jelly, oils, etc., undergoing sterilization. The 200-ml Erlenmeyer (Pyrex) flask (Fig. 49) is ideally suited as a container for petrolatum and other oils normally liquid at room temperature.

Whatever the circumstances may be, an attempt should be made to limit the quantity of oil to a  $\frac{1}{4}$ -inch layer, 1 to 1½ oz, in order to provide some margin of safety in sterilization. If larger quantities are required, then it is necessary to determine beforehand the rate of heating of each quantity of oil that deviates appreciably in volume from this condition. Failing this, it is next to impossible to establish a minimum safe exposure period, especially where the operator must rely upon the oven temperature indicated by the thermometer as the beginning of the exposure period.



Fig. 49.—The 200-ml flask is ideally suited as a container for petrolatum and other oils normally liquid at room temperature. The quantity of oil should be limited to a  $\frac{1}{4}$ -inch layer in order to provide some margin of safety in sterilization. Gauze strips impregnated with petroleum jelly can be conveniently prepared in the catheter tray. The strips should be limited to about twenty and the petroleum jelly to 4 oz, making a layer  $\frac{1}{2}$ -inch deep in the tray. (Courtesy of American Sterilizer Company.)

*Petrolatum Gauze.*—The preparation and sterilization of petrolatum (lubricated) gauze has long been a troublesome procedure. Observations in many hospitals have revealed the all too frequent error of preparing too much material, with large excess of petrolatum, inadequate exposure for sterilization or over-exposure due to heating at too high a temperature with consequent charring or partial destruction of the gauze strips.

The procedure can be easily controlled within safe limits if attention is given to the simple details of preparation and heating.



A satisfactory procedure is to first prepare about 20 strips of bandage gauze, each 6 to 8 inches long and 2 inches wide. These strips should be placed in a stainless steel catheter tray with dimensions of  $2\frac{1}{2}$  inches wide  $\times$  8 inches long  $\times$   $1\frac{1}{4}$  inches deep. Cover the strips with 4 oz of petroleum jelly, previously liquefied by heating. This should form a layer  $\frac{1}{2}$  inch deep in the tray with a thin layer of oil over the topmost gauze strip (see Fig. 49). Sterilize in the hot air oven at  $160^{\circ}\text{C}$  for  $2\frac{1}{2}$  hours.

With this material a somewhat longer period of exposure is required than in the case of oils, due principally to the increased depth of the layer in the tray. If the temperature is maintained within the range of  $160^{\circ}$  to  $166^{\circ}\text{C}$  ( $320^{\circ}$  to  $330^{\circ}\text{F}$ ) there should be no evidence of charring or discoloration of the gauze. Repeated tests have shown that a temperature of  $170^{\circ}\text{C}$  ( $340^{\circ}\text{F}$ ) will produce some discoloration, while a temperature of  $182^{\circ}\text{C}$  ( $360^{\circ}\text{F}$ ) for a short period of time produces definite charring of the gauze.

*Instruments* (Cutting Edge).—Selection of a satisfactory method for the sterilization of cutting edge instruments frequently poses a problem for the operating room supervisor. Chemical disinfection is quite commonly resorted to, particularly for scalpel blades, but great care must be used to insure that the instruments are thoroughly cleaned before immersion in the germicide and that sufficient time be allowed for disinfection to take place. Also, if the germicide contains formaldehyde or any other irritating substance, it is necessary to rinse the instruments in sterile water and dry them under sterile conditions before use. In many instances it is apparent that chemical disinfection methods are employed for convenience rather than bacteriological efficiency or surgical safety.

Dry heat sterilization of sharp instruments can conveniently be carried out in a hot air oven at  $160^{\circ}\text{C}$  for not less than one hour. The first requirement is that the instruments be clean, free from oil or grease, otherwise this exposure period may be inadequate. It is also desirable to place the sharp instruments on shallow metal trays in the sterilizer chamber so as to enhance the rate of heating and to take advantage of the heat conducting properties of the metal.

*Syringes*.—Dry heat is the method of choice for the sterilization of syringes, principally because all moisture is eliminated, thereby minimizing erosion of the ground glass surfaces. The more common method is to remove the plunger from the barrel and wrap the barrel and plunger together in one muslin cover as shown in figure 38. If desired, the needle can be embedded in gauze and sterilized with the syringe. With this method of preparation the period of exposure should be not less than 1 hour from the time

that the oven shows 160° C. Care should be used, however, to insure that the chamber is not overloaded and that some space is provided between the wrapped syringes for free air circulation.

Another method which has certain advantages is to assemble the plunger in the barrel with the needle attached. The assembled syringe is then placed in a Pyrex test tube of such diameter that

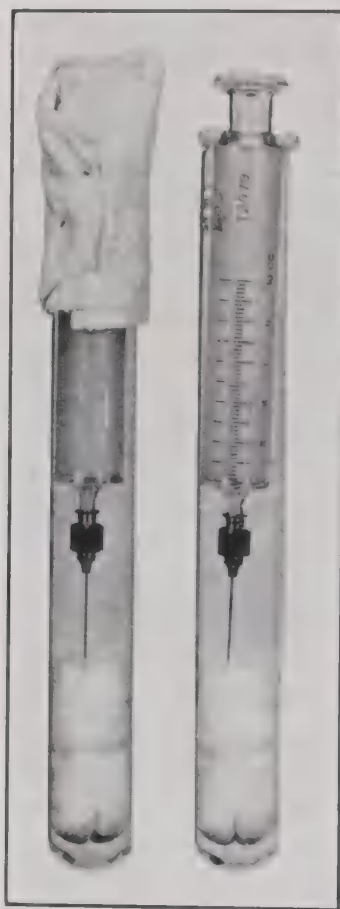


Fig. 50.—This method of preparing syringes for hot air sterilization has the advantage of a completely assembled unit with needle attached. The syringe fits loosely in the test tube with the flange resting on the rim or lip of the tube. (Courtesy of American Sterilizer.)

the barrel of the syringe fits loosely in the tube with the flange resting on top of the tube as illustrated in figure 50. The tube should be of sufficient length to accommodate the syringe with needle attached, without the point of the needle touching the bottom of the tube. (A 10-ml syringe requires a Pyrex test tube of 200 mm in length and 25 mm outside diameter, whereas a 5-ml syringe requires a tube of 150 mm in length and 20 mm outside diameter.) With this method the cotton stopper is eliminated because a portion

of the syringe extends above the rim of the tube. The upper portion of the tube should, however, be covered with a square of muslin. In this method of preparation the syringe lags the temperature of the oven by approximately 10 minutes. Therefore, it is recommended that the exposure period be not less than 75 minutes from the time that the oven shows  $160^{\circ}\text{C}$ .

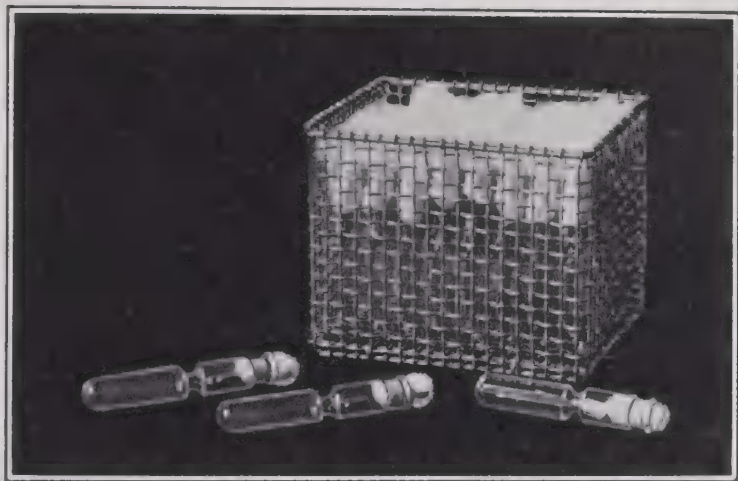


Fig. 51.—Hypodermic needles in special test tubes with restricted sides, stoppered with cotton, are ideally sterilized by means of dry heat. (Courtesy of American Sterilizer Company.)

*Needles (Hollow).* — Although hypodermic needles can be sterilized satisfactorily in the autoclave, it is much better practice to sterilize them in the hot air oven because it leaves the needles absolutely dry and assures sterility of the bore of the needle, even when the stylet is left in place. Routinely needles should not be sterilized with stylets in place because the electrolytic action set up between the stylet and needle may cause early corrosion and weakening.

A large number of needles can be sterilized at one time in the metal baskets shown in figure 51. A special test tube is used with restricted sides as indicated in the cut for suspension of the needle to protect the point. The open end of the test tube is stoppered with a cotton plug. Caution must be exercised in establishing an exposure period for a fully loaded basket of tubes. Test data indicate that the temperature of the needles in the center tubes of the basket lags considerably the temperature of the sterilizer. In fact, when the oven shows  $160^{\circ}\text{C}$  the temperature of the needle in the most centrally located tube is only  $136^{\circ}\text{C}$ . Therefore, if the period of exposure is established from the time that the oven shows  $160^{\circ}\text{C}$  it is necessary to maintain this temperature for 2 hours.



**Needles (Suture).**—When surgeons' needles are sterilized in pressure steam there is no question about sterility, but frequently serious rusting occurs which is difficult to explain. To avoid all rusting of needles, the hot air method of sterilization is recommended. For this procedure, sew the needles into a gauze pack and wrap with muslin as in figure 52. Sterilization may then be accomplished at a temperature of 160° C for not less than 1 hour.

TABLE 114.—SUMMARY OF REQUIREMENTS FOR HOT AIR STERILIZATION OF SUPPLIES (FROM PERKINS, 1951)

Material	Exposure period from time sterilizer shows temperature of			Quantity and preparation
	170° C (340° F)	160° C (320° F)	140° C (285° F)	
Glassware		60 minutes		Items must be clean and free from oil or grease.
Glycerine	60 minutes	120 minutes		Quantity should be limited to $\frac{1}{4}$ " layer (approx. 1 oz) in 200-ml Erlenmeyer flask.
Instruments (cutting edge)		60 minutes		Instruments must be clean, free from oil or grease and placed on metal tray in sterilizer.
Needles (hypodermic)		120 minutes		Needles may be placed in tubes having restricted sides, with cotton stoppers. Wire mesh baskets serve well as containers for tubes. Remove stylets.
Needles (suture)		60 minutes		Sew needles into gauze pack, wrap in muslin.
Oils	60 minutes	120 minutes		Quantity should be limited to $\frac{1}{4}$ " layer (approx. 1 oz) in 200-ml Erlenmeyer flask or similar container.
Petrolatum—liquid	60 minutes	120 minutes		Same as for Oils.
Petroleum jelly	60 minutes	120 minutes		Quantity should be limited to $\frac{1}{4}$ " layer (approx. 1 oz) in Petri dish, ointment jar or other similar container.
Petroleum jelly gauze		150 minutes		Quantity should be limited to 20 strips of 2" × 8" gauze and no more than 4-oz petroleum jelly in catheter tray with dimensions of 2½" × 8" × 1½".
Powders	60 minutes	120 minutes		Quantity should be limited to $\frac{1}{4}$ " layer (approx. 1-oz) in Petri dish or other container.
Sulfonamide powders			3 hours	Quantity should be limited to 4 to 5 g in double envelopes or cotton-plugged test tube.
Syringes (in test tubes)		75 minutes		Place assembled syringe with needle attached in test tube of suitable size. Cover top of tube with muslin.
Syringes (wrapped)		60 minutes		Remove plunger from barrel and wrap in muslin. The needle embedded in gauze may be included in pack.
Zinc peroxide			4 hours	For clinical application, quantity should be limited to 15 to 20 g in suitable container.

**Timing the Exposure.**—Close regulation of temperature is important in hot air sterilization to avoid over-exposure of heat-sensitive articles and to prevent under-exposure with the possibility of an unsterile load. After placing the load in the chamber, the heat should be turned on and the thermostat set to maintain the desired temperature range. Time the exposure only when the ther-

nometer indicates the desired range. Avoid opening the door during the exposure period as the oven will cool rapidly.

For efficient operation of any hot air sterilizer, the characteristics of the individual unit must be known. If masses of material are to be sterilized or if the methods of preparation deviate greatly from those summarized in Table 114, then it is incumbent upon the operator to determine experimentally the time required for the material to reach sterilizing temperature.

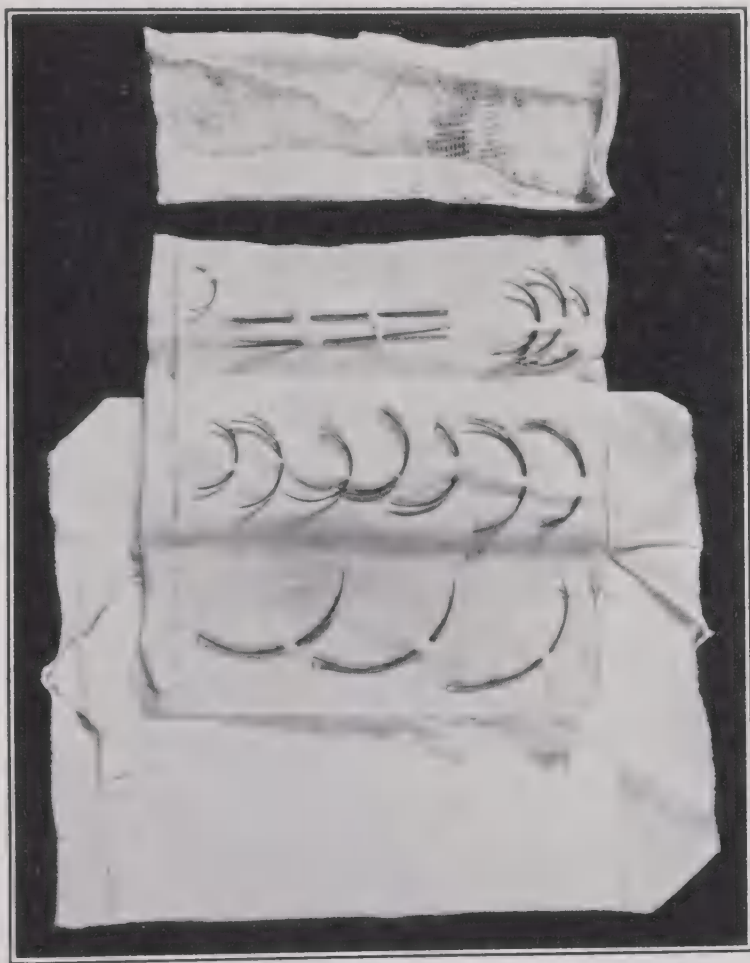


fig. 52.—Suture needles prepared in a gauze pack and wrapped in muslin may also be sterilized in the hot air oven. (Courtesy of American Sterilizer Company.)

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## 32

# THERMAL RESISTANCE OF MICROORGANISMS

### INTRODUCTION

THE resistance of many microorganisms to heat far exceeds that of any other type of life. Procedures for sterilization by heat are designed and required mainly to eliminate the microscopic forms of life known as yeasts, molds, bacteria and their spore forms. The principles of adequate and effective sterilization by heat are governed by those biological properties of microorganisms which determine and control their thermal resistance, and the development and application of the principles of sterilization by heat must arise from and be based upon the best and most complete knowledge of this phase of microbiology.

### DEATH OF BACTERIA AND SPORES

The only single practical criterion of the death of microorganisms is the failure to reproduce when, as far as is known, suitable conditions for reproduction are provided. This means that any organism which fails to show evidence of growth when placed under what are considered, in the light of our present knowledge of bacterial nutrition and growth requirements, adequate growth conditions is considered as dead. Although the external or environmental factors which can be responsible for death of a cell have been very extensively studied from both a practical and theoretical viewpoint and much is known about the external conditions which may lead to the death of microorganisms as defined above, little is known as to the intimate physical, chemical or metabolic factors within the cell responsible for the existence of this condition of the cell. Possible mechanisms of death will be discussed later.

In general the influences causing death might be divided into chemical and physical. The physical influence with which we are at present concerned is heat, and all further discussion of bacterial death will be made with reference to the effect of this factor, although there is no doubt that a thorough interweaving of all existent knowledge of the death of microorganisms whether by physical or chemical means, if it could be accomplished, might result in a better understanding of the phenomenon described as the death of microorganisms.

Much of the early work on death by heat, or heat resistance of microorganisms was done by means of a "thermal death point" determination. In this procedure samples of a culture or suspension were exposed for a fixed time period, usually 10 minutes, to various temperatures and survival or no survival determined either by direct incubation if the suspending medium were suitable for growth, or by subculture into a suitable medium. The "thermal death point" was the lowest temperature which resulted in no survival after the fixed period of exposure adopted. While it seems to have been realized that "thermal death point" could depend upon the concentration of viable cells, the nature of the suspending medium, age and physiological condition of the cells and many other factors, few attempts were made in work of this type to adopt or specify standard conditions. Much of the early work on "thermal death point" has been summarized in a tabulated form by Hampill (1932).

Parallel with experiments upon "thermal death point" were determinations of thermal death time. Due to the long survival of spores at temperatures killing vegetative cells, the procedure was revised and the time required to kill the spores when subjected to various temperatures was determined. Globig (1881) is cited by Buchanan and Fulmer (1930) as apparently the first to make critical studies of the death of bacterial spores under the influence of heat. The early data obtained upon this subject again are influenced by lack of consideration of cell concentration, suspending medium, and other factors which are now known to have a definite influence upon thermal death time.

The first modern critical approach to the problem appears to be that of Bigelow and Esty (1920). They start with a definition, "By thermal death point in relation to time is meant the time at different temperatures necessary to completely destroy a definite concentration of spores in a medium of known hydrogen-ion concentration." The thermal death point in relation to time, which soon became known as thermal death time was determined by exposing a known number of spores suspended in a specified medium



in sealed glass tubes to heat treatment in an accurately controlled oil bath. Tubes were removed from the bath after different times of exposure and the contents transferred to a nutrient medium to determine survival or death. Thermal death point in relation to time or thermal death time as it will be hereafter expressed was conceived to lie between the longest time showing survival and the next longer exposure time which naturally was the shortest time showing no survival. The principle of specifying thermal death time as lying between a maximum survival time and a minimum destruction time has been fundamental in almost all modern work upon the thermal resistance of microorganisms although there have been some marked advances in techniques of the determination in more recent years, and recently attempts have been made to express thermal resistance in terms of destruction rates rather than destruction time.

Using the concept of thermal death time and the technique as described, Bigelow and Esty (1920) demonstrated that with any given spore suspension, the thermal death time was a function of the spore concentration, and that thermal death time for a given spore concentration decreased with increase in temperature. The value of the method in comparing the thermal resistance of the spores of different organisms was amply demonstrated by the data presented.

Bigelow (1921) using the same data pointed out the logarithmic relationship between temperature and thermal death time. When the survival and destruction points for any given suspension and spore concentration were plotted upon a logarithmic scale against the corresponding temperatures on a linear scale, essentially straight lines were obtained.

Immediately following the pioneer work of Bigelow and Esty (1920) extensive determinations of the resistance of spores of *Clostridium botulinum* were carried out in several different laboratories, Weiss (1921a, 1921b), Esty and Meyer (1922), Esty (1923), Sommer (1930), and others. The net result of this work was to establish the maximum thermal death time of a large inoculum (60 billion spores) of the most highly resistant strain of *Cl. botulinum* which has ever been encountered. The thermal death time of this organism at different temperatures in phosphate buffer at pH 7.0 provides the data for what is considered the "classic" resistance of *Cl. botulinum* and has remained a standard of reference, with minor correction (Townsend, Esty and Baselt, 1938) since that time.

Esty and Williams (1924) called attention to the occurrence of "skips" when single tubes were removed at each time interval and suggested a multiple tube procedure for eliminating this source

of error or uncertainty. It was found that single tubes exposed for several consecutive time intervals might show an indication of destruction at one time of exposure and survival at a longer time of exposure. Esty and Williams showed that if a number of tubes were heated at the same time intervals, at some exposure times only a certain percentage of the total tubes would show survival. If a number such as 25 tubes were subcultured after various heating times, at the shortest heating time all would show survival and as heating time increased the percentage of tubes showing survival decreased in a rather regular manner until all tubes showed destruction. The percentage survival plotted on a logarithmic scale against time on a linear scale gave an approximation to a straight line. Based on these results a new method of evaluation of thermal death time was suggested. Although the original suggestion was never carried out and it is doubtful whether there is or can be a strict logarithmic relationship between exposure time and the percentage of positive tubes, the basic principle of multiple samples in order to avoid "skips" was adopted in almost all subsequent work.

Although Bigelow (1921) had shown the semi-logarithmic relationship between destruction time and temperature and plotted the thermal death time curve, almost all reports on thermal resistance of spores until the time of Townsend, Esty and Baselt (1938) seem to have been presented as tabulations of survival and destruction times, rather than using the demonstrated relationship to characterize thermal resistance. Ball (1923, 1928) in developing the mathematical methods used for the calculation of processes for canned foods pointed out that the thermal death time curves of Bigelow (1921) could be characterized by a point and a slope. The reference point chosen was the time to destroy the organism at 250° F and this was designated by the symbol  $F$ . The slope of the thermal death time curve symbolized by  $z$  was defined as the number of degrees required for the curve to traverse one log cycle. This is the equivalent of the number of degrees the temperature must be raised or lowered from a given reference temperature to produce a tenfold decrease or increase in destruction time.

Townsend, Esty and Baselt (1938) adopted the definitions of Ball and established their use rather firmly for purposes of characterizing thermal resistance when an endpoint or survival-destruction technique is used. These authors also proposed certain conventions in the plotting of thermal death time curves from survival-destruction data which have been rather generally adopted by workers in this field. These conventions were expressed as follows:

1. A survival point is considered as positive data and the curve must be above (higher in temperature or longer in time) than every survival point.

2. Destruction points are indicative but not positive, owing to the phenomenon of skips (survival of organisms at a time beyond that at which sterility is indicated). In general, however, a thermal death curve should be beneath as many destruction points as possible and still be above all survival points.
3. The slope of the thermal death time curve should be parallel to the general trend of the survival and destruction points.

The authors also point out that if only two temperatures are used the curve becomes very ambiguous since many curves can be drawn between the survival and destruction points with different slopes. Therefore at least three and preferably more temperatures should be used in order to determine a valid and useful thermal death time curve.

Figure 53 shows a thermal death time curve drawn in accordance with the conventional method as outlined by Townsend, Esty and Baselt (1938). The data used were obtained with a 10,000 spore inoculum of PA 3679 in beef and gravy, run in thermal death time cans with product incubation (unpublished data from this laboratory). This is almost an ideal curve, as a straight line can be drawn above every survival point and below or through every destruction point. For this curve  $F = 9.5$  and  $z = 20$ . Many curves will not fit the data quite as closely as this, although they may tend to do so as the number of samples heated at each time interval is increased.

In addition to explicit definitions and criteria for establishing the thermal death time curve, Townsend, Esty and Baselt reported new procedures for the determination. Previous work had all been carried out in sealed tubes with an oil bath as the heating medium. These workers described the use of steam as a heating medium for sealed tubes or "thermal death time cans." The procedure depends upon the use of small, accurately controlled steam retorts and small cans ( $208 \times 006$ ) which can be heated rapidly to the processing temperature with a minimum requirement for correction for lag in temperature. The equipment used was described by Townsend, Esty and Baselt (1938) and by Pilcher *et al.* (1947). The sealed tubes were subcultured into media as in previous work but the thermal death time cans were incubated after processing. This provided a means to obtain product incubation tests of heated spores which might resemble conditions of food processing.

Since that time two further procedures for the determination of thermal death time have been described. Stumbo (1948a) described the principles and construction of the "thermoresistometer." The equipment is designed to heat small amounts of suspension



and substrate (0.01 ml of each) to high temperatures without appreciable lag in temperature and to cool as rapidly as possible. Correction for come-up and cooling are negligible in this procedure.

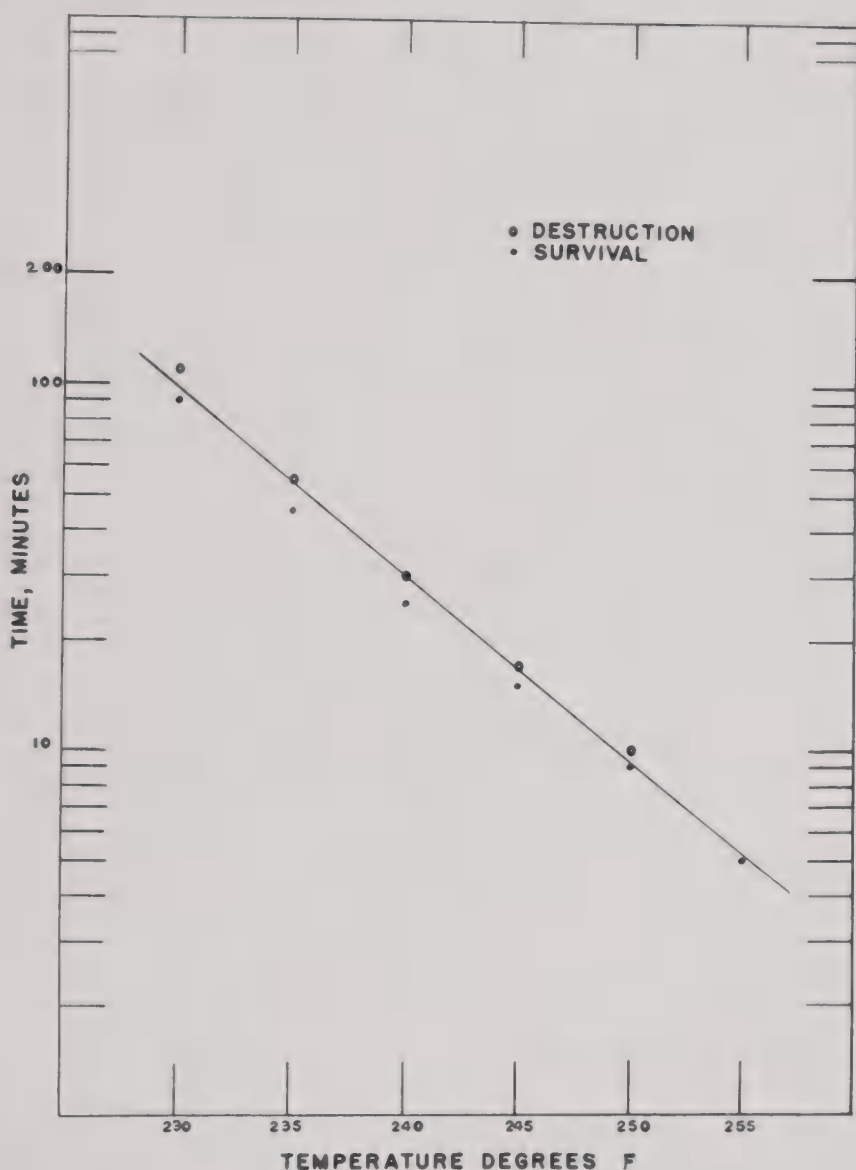


Fig. 53.—Thermal death time curve, log survival and destruction time plotted against temperature.

This permits determinations at high temperatures (260° to 270° F) where thermal death times are very short. The apparatus also is equipped for automatic timing and movement of the container through the heating chamber, which is necessary for the very short time intervals of exposure required at high temperatures. Schmidt

(1950) described a procedure for heating 13 × 100 mm cotton plugged tubes in steam and cooling under pressure which permitted subculture by the addition of the medium to the suspension in the tube after heating, rather than the transfer of the suspension to a subculture medium with possible attendant loss of viable organisms. Although a specially constructed "miniature retort" was developed for this purpose, since that time the retort system used for thermal death time cans has been successfully modified and adapted for use with "plugged tubes."

The procedures described to this point have been essentially directed to the determination of the "survival" and "destruction" times for some given initial spore concentration. The basic pre-supposition of this work often not explicitly acknowledged is that there is a time for a given temperature and suspending medium for any initial spore concentration of a given organism at which all of the organisms are dead. Attention will now be turned to an alternative means of determining the thermal resistance of microorganisms.

Very early in the studies of the death of microorganisms under adverse influences attention was given to the number of survivors remaining in relation to time, and attempts were made to measure the "rate of death" of bacterial suspensions, exposed to some adverse environmental factor such as chemicals or high temperature. This was accomplished by the use of plating procedures to determine the original number of organisms present and the number of viable organisms remaining after various periods of exposure. The rate of destruction curve or survivor curve was then obtained by plotting the number of survivors against time of exposure. For temperatures not exceeding 212° F the suspensions are exposed in a suitable container in a water bath with or without stirring and samples removed with a pipette at designated intervals of time, or individual tubes so exposed are removed and sampled. A great many modifications of this technique have been used, Chick (1910), Ayers and Johnson (1914, 1915), Baker and McClung (1939), Beamer and Tanner (1939*a*, 1939*b*) to cite only a few. For temperatures higher than 212° F a similar procedure has been used by placing suspensions in sealed tubes and exposing them in a bath of oil or other heating medium. One or replicate samples are removed at designated intervals and the number of survivors determined (Gillespy, 1946, 1947, 1948; Sugiyama, 1951; Reynolds and Lichtenstein, 1952).

Williams, Merrill and Cameron (1937) described the "tank" method for the determination of spore destruction rates at temperatures above 212° F. The equipment permits heating a suspen-

sion in any liquid, or in mixture of liquid and suspended solids, under pressure with constant stirring and the removal of samples at any time desired while continuing the exposure of the remainder of the material. The time required to bring the "tank" to temperature is comparatively short and then uniform heating conditions are established. This apparatus permits the study of spore destruction rates under conditions as they might exist within a can of food being processed.

Madsen and Nyman (1907), and Chick (1908, 1910) independently and almost simultaneously reached the conclusion that the rate of death of microorganisms showed a logarithmic order, that is the number of surviving organisms plotted upon a logarithmic scale against time on a linear scale gave a straight line. The data upon the logarithmic order of death of microorganisms under various influences has been extensively reviewed and discussed by Rahn (1932, 1943, 1945*a*, 1945*b*). Additional evidence for a logarithmic order of death may be found in recent publications of Gillespy (1946, 1947, 1948), Olsen and Scott (1950), Murrell, Olsen and Scott (1950), while the survivor curves in experiments recently published by Sugiyama (1951) also appear to be approximately logarithmic in nature. Early evidence for a non-logarithmic order of death has been reviewed by Knaysi (1930*a*, 1930*b*, 1930*c*), Rahn (1930, 1932, 1943, 1945*a*, 1945*b*). Recently a non-logarithmic order of death of spores of food spoilage organisms has been reported by Anderson *et al.* (1949), Reed, Bohrer and Cameron (1951), and Reynolds and Lichtenstein (1952).

The nature of the curve which describes the relation between number of survivors and time of exposure is still an open question and the writer ventures to believe it is one which will long haunt the bacteriologist concerned with sterilization problems. While some workers, Rahn (1945*b*), Baumgartner (1949), Gillespy (1951), Stumbo (1948*a*, 1948*b*, 1949*a*, 1949*b*) and others, impressed by the weight of evidence for a logarithmic order of death, appear willing to accept it as the indisputable standpoint from which all further discussion must procede, the writer would like to suggest the following viewpoint. The evidence in favor of a logarithmic order of death is considerable and impressive and warrants the full exploitation of the consequences of such order in application to experimental data. Evidence of the finding of a non-logarithmic order of death is also at hand. This must be judged to some extent in the light of the experimental difficulties in determining survivor curves. Where the data show a close approximation to a logarithmic order, there is a strong presupposition that this is the case; where the data deviate there should be sufficient data to show that the



curve in question is statistically incompatible with a logarithmic order. From this point of view a logarithmic order may be assumed as a working hypothesis where there is no contrary evidence at hand, with the danger always present that discrepancies will result if the

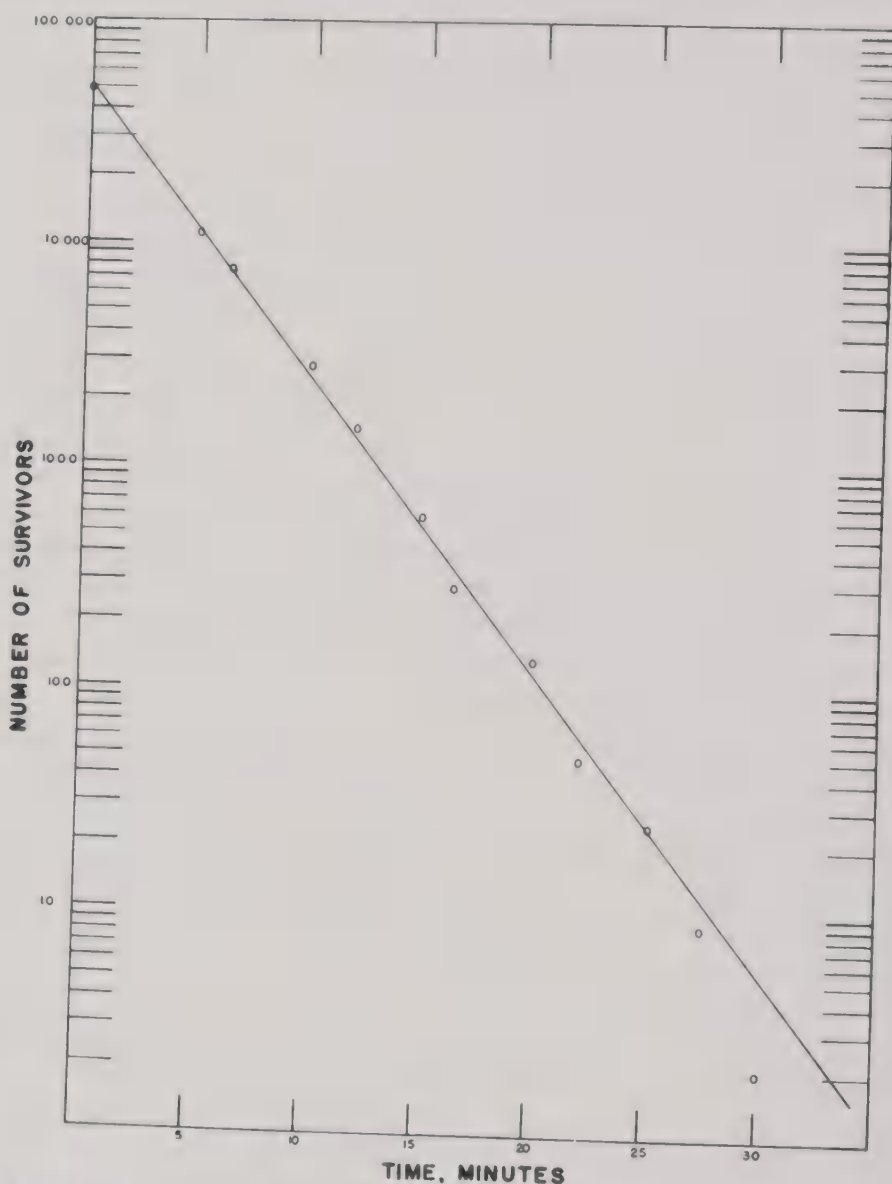


Fig. 54.—Survivor curve, log survivors plotted against time of exposure.

case under consideration does not follow a logarithmic order. Furthermore the application of concepts based on a logarithmic order to cases of a non-logarithmic order will be in error to the degree that the particular case deviates from logarithmic, but in many cases the advantages obtained may outweigh the errors encountered.

With these remarks in mind the discussion may be turned to the consideration of the curve which describes a logarithmic order of death.

Figure 54 shows a curve plotted from data of Williams, Merrill and Cameron (1937) for the destruction of spores of a thermophilic flat sour organism (N.C.A. #1518) at 248° F in phosphate buffer. The number of survivors is plotted upon a logarithmic scale against time on a linear scale. These data show a logarithmic rate of destruction, since the points give a good fit for a straight line. This curve may be described by the equation:

$$K = \frac{1}{t} \left( \log N_0 - \log N \right)$$

where  $K$  = a constant, depending upon the organism, temperature, substrate, and assuming logarithms to base 10.

$t$  = time of exposure in minutes.

$N_0$  = number of organisms present at beginning of the time interval.

$N$  = number of organisms present at the end of the time interval.

From figure 54 taking  $N_0 = 50,000$ ,  $t = 5$  minutes,  $N = 12,000$

$$K = \frac{1}{5} \left( \log 50,000 - \log 12,000 \right) = \frac{4.69 - 4.08}{5} = 0.122.$$

The curve illustrated in figure 54 may be called the survivor curve for the organism and conditions concerned and is characterized by the numerical value of  $K$ . In most work involving thermal resistance studies the velocity constant  $K$  is a very small number and is cumbersome to use and write. Therefore simpler constants mathematically related to  $K$  have been introduced. Baker and McClung (1939) measured the time required to reduce bacteria to 0.01 per cent of their original number. Katzin, Sandholzer and Strong (1943) pointed out that a reduction of the original number to 10 per cent of the original number gives the equation:

$$(2) \quad K = \frac{1}{t} \log \frac{N_0}{0.1N_0} \text{ or } K = \frac{1}{t} \text{ or } t = \frac{1}{K}$$

The time  $t$  at which there are 10 per cent survivors or 90 per cent reduction of the original number they defined as the Decimal Reduction Time (D.R.T.). It should be noted that D.R.T. in addition to being the reciprocal of  $K$  is also the time required for the survivor curve to pass over one log cycle if  $\log N$  is plotted against time. Ball (1943) used  $Z$ , following a suggestion of Baselt, to designate

the time for the survivor curve to pass over one log cycle. Stumbo (1948a, 1949a, 1949b) has used  $Z$  in the same sense. Gillespy (1946, 1947, 1948) has used the terms Decimal Reduction Time, D.R.T. and  $D$ . In view of the possible confusion between  $Z$  and  $z$  = slope of the thermal death time curve which has been firmly established in the literature much longer (Ball, 1923), the writer suggested in 1950 that  $D$  be adopted as a symbol to designate the factor

variously expressed as Decimal Reduction Time,  $Z$  or  $\frac{1}{K}$ . Such usage has been adopted recently (Reed *et al.*, 1951). Since  $D$  may be determined and expressed for any temperature a sub-script may be used to designate temperature such as  $D_{250}$ ,  $D_{240}$ , etc.

TABLE 115.—THERMAL RESISTANCE DATA FOR 1000 SPORES PER CAN OF THERMOPHILIC FLAT SOUR ORGANISM #617 AT 250° F IN WHOLE MILK, RUN IN THERMAL DEATH TIME CANS WITH PRODUCT INCUBATION

Corrected heating time, minutes	Number of containers		
	Heated	+	—
2.0	10	9	1
2.5	10	7	3
3.0	10	1	9
3.5	10	2	8
4.0	10	0	10

Ball (1943) gave a summary of the methods used for the calculation of processes for canned foods based upon heat penetration data and the thermal death time of *Cl. botulinum* or significant spoilage organisms. Ball suggests obtaining the "phantom thermal death time curve" by plotting  $Z = D$  values obtained from survivor curves at different temperatures, upon a logarithmic scale against temperature upon a linear scale. This was considered a phantom curve since it was supposed to have direction but no position. The slope of this curve measured in degrees required to pass over one log cycle was equal to  $z$  in the same sense as  $z$  of the thermal death time curve.

$D$  may be calculated from survivor curves where available as suggested by Ball (1943) and as has been done by Gillespy (1946, 1947, 1948).  $D$  may also be calculated from the results of multiple sample thermal death time determinations when all the data are taken into consideration. In most cases of thermal death time determinations where multiple samples are run at successive time intervals with reasonably close spacing, data of the type shown in table 115 are obtained. These results obtained in whole milk with a



thermophilic flat sour organism heated and incubated in thermal death time cans will be used for purposes of illustrating the various types of calculation of  $D$  values.

TABLE 116. — CALCULATION OF  $D$  FROM DATA IN TABLE 113 ACCORDING TO THE PROCEDURE OF STUMBO (1948a)

Time	Tested	Number of containers		$\log A$	$B$	$\log B$	$D$
		+	—				
2.0	10	9	1	4.0	9	0.95	0.66
2.5	10	7	3	4.0	7	0.85	0.79
3.0	10	1	9	4.0	1	0.0	0.75
3.5	10	2	8	4.0	2	0.3	0.95
4.0	10	0	10	4.0			
							Ave. 0.79

Stumbo (1948a) used the formula:

$$Z \text{ or } D = \frac{U \text{ or } t}{\log A - \log B}$$

where  $A$  was the total number of samples heated multiplied by the number of spores per sample.

$B$  was calculated by assuming one surviving spore per container when less than the total number of containers showed survival.

$U$  or  $t$  was exposure time at a given temperature.

The calculation of  $D$  according to this method is given in table 114.

Stumbo, Murphy and Cochrane (1950) use the same formula for the calculation of  $D$ , but calculate  $B$  the number of survivors by means of the "most probable number" procedure of Halvorson and Ziegler (1933) where

$\bar{x}$  = most probable number of spores surviving per replicate

$n$  = total number of replicates

$q$  = number of *sterile* replicates

$$\text{and } \bar{x} = 2.303 \log \frac{n}{q}$$

then  $B = \bar{x}$  times number of replicates

Calculation of  $D$  for the same data by this procedure is shown in table 117. The procedures of Stumbo (1948a) and Stumbo, Murphy and Cochran (1950) call for averaging the  $D$  values for each exposure time, as shown in tables 114 and 115.

The writer several years ago developed a method for evaluating thermal resistance based on a procedure for bioassay suggested by Reed (1936). In this procedure two assumptions are made: first that any sample not showing survivors at a given exposure time, would not show survivors at a longer time of exposure, second that any sample showing survivors at a given exposure time would show

TABLE 117.—CALCULATIONS OF *D* FROM DATA IN TABLE 113 ACCORDING TO PROCEDURE OF STUMBO, MURPHY AND COCHRANE (1950).

Time	Tested	Number of Containers		<i>log A</i>	<i>q</i>	<i>n/q</i>	<i>log n/q</i>	<i>x/</i>	<i>B</i>	<i>log B</i>	<i>D</i>
		+	—								
2.0	10	9	1	4.0	1	10	1.0	2.3	23	1.36	0.76
2.5	10	7	3	4.0	3	3.33	0.52	1.2	12	1.08	0.85
3.0	10	1	9	4.0	9	1.11	0.046	0.106	1.06	0.02	0.80
3.5	10	2	8	4.0	8	1.25	0.098	0.225	2.25	0.35	0.96
4.0	10	0	10	4.0							

Ave. 0.84

survivors at a shorter exposure time. These two assumptions permit a cumulative test to be constructed from the actual data as shown in table 118, where column *m* shows the cumulated samples surviving each exposure time obtained by adding the positive samples upward from the longest exposure time to the shortest, and column *n* shows the cumulated samples not surviving each exposure time, obtained by adding the negative samples downward from the shortest exposure time to the longest. The probability of sterility at any exposure time is then calculated from the formula:

$$P = \frac{n + 1}{m + n + 2}$$

TABLE 118.—CALCULATION OF *D* FROM DATA IN TABLE 113 ACCORDING TO THE PROBABILITY PROCEDURE

Time	Tested	Number of Containers		<i>m</i>	<i>n</i>	<i>m + n</i>	<i>P</i>
		+	—				
2.0	10	9	1	19	1	20	.09
2.5	10	7	3	10	4	14	.31
3.0	10	1	9	3	13	16	.78
3.5	10	2	8	2	21	23	.88
4.0	10	0	10	0	31	31	.97

$$D = \frac{2.8^*}{3.0 + 0.16} = 0.89$$

\* L. D. 50 read from Figure 55

Using arithmetic probability paper the probability of sterility is plotted against exposure time and a straight line fitted to the points. The time read from the curve corresponding to  $P = 0.50$

has been called the L.D. 50 point in analogy with the treatment of bioassay data. This point represents the time, as determined from the cumulative test using all the data, at which 50 per cent of tubes will be sterile. Since this point or time corresponds to 0.69 surviving organisms per tube, Halvorson and Ziegler (1933),  $D$  is calculated in the following manner:

$$D = \frac{\text{L.D. 50}}{\log A - \log 0.69} \quad \text{or} \quad \frac{\text{L.D. 50}}{\log A + 0.16}$$

where  $A$  = the initial number of organisms per tube.

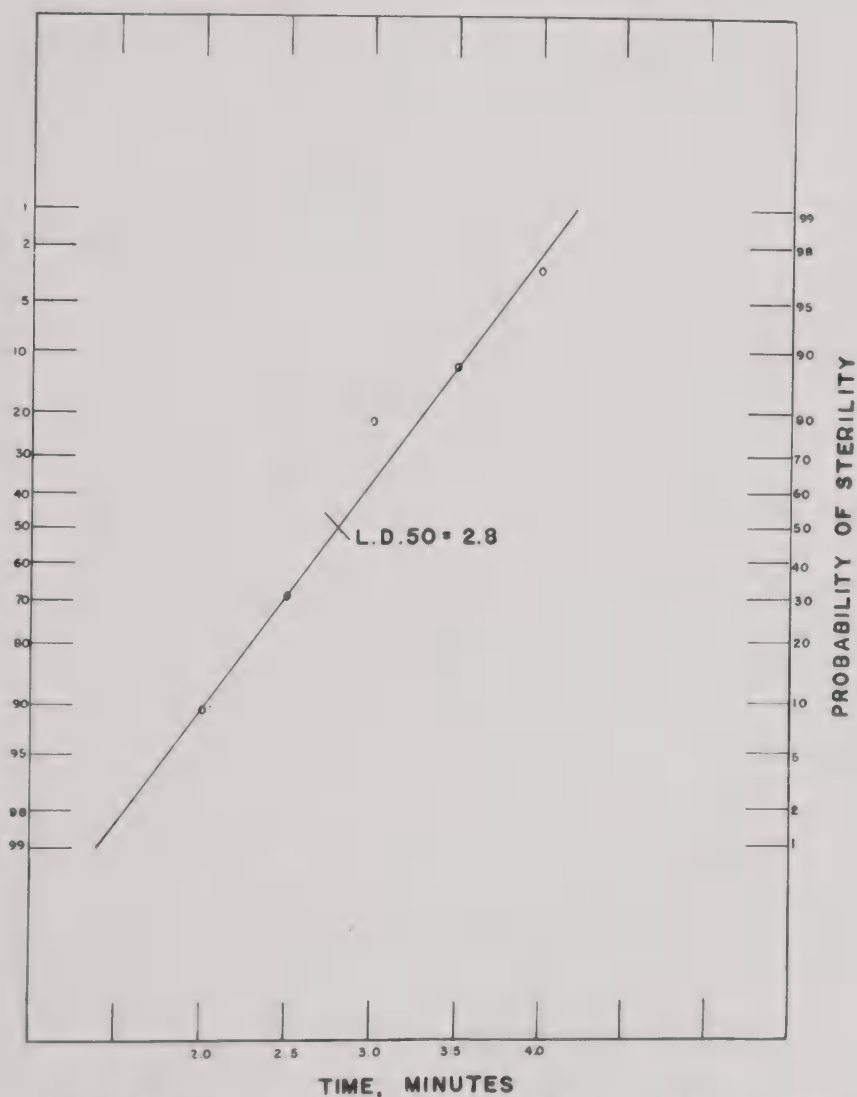


Fig. 55.—Probability curve, probability of sterility plotted against time.

Figure 55 shows the probability of sterility plotted against time from the data in table 115. The calculation of  $D$  for these data is shown beneath table 118.



Reynolds *et al.* (1952) have published a brief description of the probability procedure with the writer's permission and have compared  $D$  calculated by this procedure with  $D$  calculated by the Stumbo (1948a) procedure. They note that "z values for the curves established by both methods were generally in close agreement." The writer has made an extensive comparison of the  $D$  values obtained by the three methods of calculation applied to a large amount of unpublished data. In general the  $D$  values calculated by the three procedures show agreement within about 10 per cent; however, from time to time the probability method will give a value significantly

TABLE 119.—COMPARISON OF  $D_{250}$  VALUES OBTAINED BY DIFFERENT METHODS OF CALCULATION

Organism	Product	Procedure	$D_{250}$ Calculated by		
			Stumbo (1948a)	Stumbo, Murphy and Cochrane (1950)	Probability Method
PA 3679	Cream style corn	T.D.T. can	2.44	2.47	2.53
PA 3679	Whole kernel corn (1)	T.D.T. can	1.48	1.52	1.54
PA 3679	Whole kernel corn (2)	T.D.T. can	1.69	1.82	1.85
PA 3679	Phosphate buffer	Tube	1.30	1.31	1.32
FS 5010	Cream style corn	T.D.T. can	1.12	1.14	1.09
	Whole kernel corn	T.D.T. can	1.28	1.35	1.38
FS 1518	Phosphate buffer	Tube	2.95	3.01	3.04
FS 617	Whole milk	T.D.T. can	0.79	0.84	0.79
	Evaporated milk	Tube	0.98	1.05	1.05

different than that obtained by other methods, this is also apparent in the tabulated data of Reynolds *et al.* (1952). More intensive study of the results of the different methods of calculation seems to be necessary before it can be said that one is preferable to another. All interested workers who have thermal death time data amenable to this type of treatment will contribute to the solution of the problem by calculating  $D$  values by the various methods. Table 119 shows a comparison of  $D$  values calculated by the three methods from unpublished data.

There have been little published data available to test the reproducibility of  $D$  values from replicate experiments upon the same suspension under the same conditions or for the same suspension

tested at different initial levels of inoculum. Reed, Bohrer and Cameron (1951) using the tank method found different  $D$  values calculated from survivor curves for three different initial inoculum levels with spores of 1518 flat sour organism, and for two different inoculum levels of spores of PA 3679 when tested in phosphate buffer. Table 120 shows representative data obtained in this laboratory on suspensions heated in phosphate buffer and subcultured using the plugged tube procedure. These results show the degree to which  $D$  values may be reproducible and that essentially the same  $D$  value is obtained when calculated from data obtained from different spore concentrations.

Stumbo (1948a), Stumbo, Murphy and Cochran (1950) and Reynolds et al., (1952) have shown that  $D$  values plotted on a logarithmic scale against temperature on a linear scale give straight

TABLE 120.— $D_{250}$  FOR REPLICATE DETERMINATIONS AND FOR VARIOUS INITIAL SPORE CONCENTRATIONS

Suspension		Spore concentration				
		100,000	10,000	1,000	100	10
FS 1518	Test 1	3.00	2.74	2.76	3.39	3.22
	Test 2	3.20	3.10	2.97	2.96	3.04
PA 3679-20*		1.13	1.12			
PA 3679-21*	Test 1	1.34	1.32			
	Test 2	1.20	1.19			

\* Different suspensions of PA 3679

lines for the relationship between  $\log D$  and temperature. This represents the "phantom" thermal death time curve according to Ball (1943) which has direction but no position. Figure 56 shows such a curve for  $D$  values of PA 3679 in beef and gravy, obtained by calculation of  $D$  from the same data used to illustrate figure 53, the thermal death time curve by the conventional method.

Olson (1950) and the writer have applied the name of thermal resistance curve to this type of curve and rather than considering it a "phantom" are inclined to think that it represents the real measure of thermal resistance. It has both direction and position since it can be characterized by point and slope by taking  $D_{250}$  and in the same sense as  $F$  and  $z$  of the thermal death time curve defined by Ball (1928). Since  $D$  is calculated from assumptions of a logarithmic rate of destruction it will be valid to the extent that logarithmic or approximately logarithmic rates of destruction take place. This viewpoint will be greatly strengthened when more data are available to show that the same thermal resistance curve can be obtained from different initial spore concentrations. In any

event the distinction between the *thermal death time curve* obtained by fitting a line thru destruction and survival points for some specific level of inoculum, and the *thermal resistance curve* obtained by fitting a line to *D* values plotted against temperature which is,

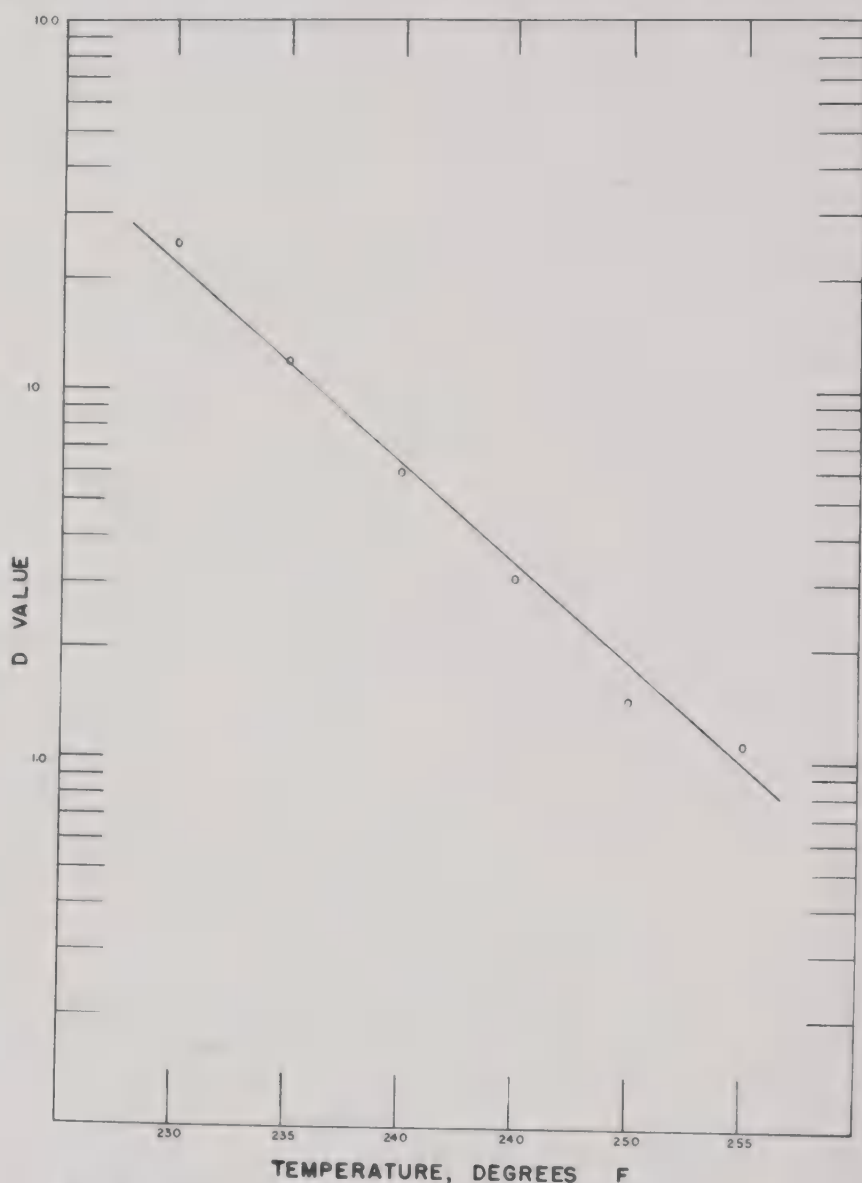


Fig. 56.—Thermal resistance curve, log *D* values plotted against temperature.

theoretically at least, independent of inoculum level, appears to be a valid distinction which should be maintained rather than referring to both curves indiscriminately as thermal death time curves.

It has been recognized since the work of Bigelow and Esty (1920) that the resistance of a suspension is dependent upon the



initial number of spores or organisms present. For this reason  $F$  values of different suspensions are not comparable unless the numbers in the initial inoculum are comparable. It is impossible therefore to compare  $F$  values in the literature when some data are reported for 10,000 to 20,000 spores and other data for 100,000 to 1,000,000 spores.  $D$  values to the extent that they are valid are directly comparable, since they are independent of concentration. A method for the interconversion of  $F$  and  $D$  therefore is highly desirable. A study of much unpublished data in which  $F$  was determined by the conventional procedure and  $D$  calculated by the probability method suggested that the following formula gave the best interconversion:

$$D = \frac{F}{\log A + 2} \quad \text{or} \quad F = D (\log A + 2)$$

TABLE 121.—COMPARISON OF  $F$  AND  $D$  VALUES AS DETERMINED EXPERIMENTALLY AND AS CALCULATED BY CONVERSION FORMULAE

<i>Suspension and spore concentration</i>	<i>Substrate</i>	<i>Temp. ° F</i>	<i>Experimental</i>		<i>Calculated</i>	
			<i>F</i>	<i>D</i>	<i>F</i>	<i>D</i>
PA 3679-10,000	Beef and gravy	235	54	11.2	67	9.0
		240	30	6.1	36	5.0
		245	17	3.2	19	2.8
		250	9.5	1.3	8	1.6
		255	5.4	1.0	6	0.9
PA 3679-10,000	Mushroom soup	235	42	5.9	35	7.0
		240	24	3.5	21	4.0
		245	13	2.3	13.8	2.2
		250	7.5	1.2	7.2	1.2
FS 617-1000	Whole milk	240	18	3.8	19	3.6
		245	8.8	1.6	8	1.7
		250	4.3	0.8	4	0.86
FS 730-100,000	Whole milk	230	39	5.8	40	5.65
		235	20	2.9	20	2.86
		240	10	1.2	8.4	1.42
FS 5230-1,000,000	Whole milk	240	19	2.7	21.6	2.4
		245	9.5	1.3	10.4	1.2
		250	4.4	0.6	4.8	0.57

Representative data for the conversion of  $D$  to  $F$  and  $F$  to  $D$  are given in table 121. The values of  $F$  at each temperature represent the reading from the thermal death time curve;  $D$  at each temperature was calculated from the spoilage data at that temperature. The conversions suggested are tentative and purely empirical. They may be expected to be valid to the degree that approximately logarithmic rates of destruction hold. Also the accuracy of the conversion will depend upon the sufficiency of the data upon which

either the  $F$  or  $D$  used for conversion is based. Much more work upon this type of calculation is necessary.

### FACTORS INVOLVED IN THE DETERMINATION OF THERMAL RESISTANCE

Prior to the work of Townsend, Esty and Baselt (1938) there appears to have been little consideration given to the corrections to be applied for the lethal effects occurring during the time required to bring the test samples to temperature or to adequate correction for the original come-up time. In most cases some arbitrary period was allowed for the samples to reach temperature and then this was either ignored or included in the total exposure time. Townsend, Esty and Baselt (1938) used unpublished correction factors determined by Richardson and by Ball, Williams and Merrill. Using these factors corrections were made for both cans and tubes in the new experimental work, and the factors were also applied to obtain a corrected curve for the ideal thermal death time curve of *Cl. botulinum* in phosphate buffer from the original data of Esty and Meyer (1922). Sognefest and Benjamin (1944) published a detailed paper upon the measurement and calculation of come-up time and correction for lethality occurring during this period. An example is given for thermal death time cans showing the large difference in  $F'$  and  $z$  depending upon whether the thermal death time curve is obtained from corrected or uncorrected values for heating time. The importance of adequate corrections for come-up are now recognized and work conducted in many laboratories during the past few years has taken the importance of this correction into consideration. However, the earlier published work will always bear some degree of uncertainty unless sufficient data and description of methods are available to permit a recalculation from established correction factors.

Each of the techniques used possesses certain advantages and disadvantages which may be briefly reviewed. The oil bath is the simplest and least expensive equipment. It has been used for sealed tubes, and Reiman (1950) used the oil bath for heating thermal death time cans. The main disadvantages are the much slower heat transmission to the containers (Sognefest and Benjamin, 1944), and lack of heating capacity, causing a drop in bath temperature if many samples are immersed at one time. The slow heat transmission causes the introduction of larger corrections for heating lag which may form a very appreciable portion of the total lethal treatment at temperatures of 250° F or higher. The thermoresistometer (Stumbo, 1948a) possesses the advantage of almost instantaneous

come-up and cooling and is adaptable to use at temperatures as high as 270° F. However, it requires the use of extremely minute samples of material, 0.01 ml each of suspension and product, and can only be adapted to product incubation tests if products are separately sterilized in subculture tubes. It is necessary also to use all products in liquid or puree form, rather than the natural condition as might exist in vegetables and brine or other products with suspended particles. Secrist *et al.*, (1952) have eliminated the dilution factor by inoculating the purees with a concentrated suspension and mixing in a blender prior to distributing the material in the cups.

Heating in steam in the small retorts gives reasonably rapid and reproducible come-up and cooling time, permitting the establishment of accurate correction factors. It is adaptable for use with cans, sealed tubes or plugged tubes. Thermal death time cans permit the use of viscous products difficult to introduce into a tube, although tubes have been used by special procedures, Yesair and Cameron (1942) Stumbo, Gross and Vinton (1945a). Cans also permit the use of products in the form as they actually occur in commercial processing, such as vegetables and brine and other products with suspended particles. Products of sufficiently thin consistency as to be distributed with a pipette may be handled in tubes as well as in cans. Cans have been used primarily for product incubation as subculture of the total contents has been difficult. The recent ingenious idea of Reynolds *et al.*, (1952) for subculturing cans by puncturing and introducing concentrated media with a syringe and needle, then solder tipping the puncture, has introduced a method whereby subculture of thermal death time cans becomes feasible, if laborious. Sealed tubes may be used either for product incubation or subculture. The plugged tube has some advantages for subculture in avoiding loss of remaining viable spores. In considering subculture the dilution of product in subculture medium may be important. In the usual procedures for subculture of sealed tubes the contents are added to 15 ml or more of medium so that dilution is about 1 to 15; due to the size of tubes used, dilution in the plugged tube procedure is about 1 to 5; in the can procedure as suggested by Reynolds *et al.*, (1952) the incubation medium is essentially that of the product supplemented by medium constituents and buffered to a desired pH. Depending upon the nutritional adequacy of substrate and medium, and inhibitors or anti-inhibitors in either substrate or medium, the results of the different procedures of subculture upon the same suspension heated in the same substrate might vary considerably.

The "tank" method for determination of thermal resistance has been used extensively only in one laboratory. A recent publica-



tion, Reed, Bohrer and Cameron (1951) has given a detailed presentation of a large amount of data obtained by this procedure. This method permits rapid heating and is adaptable to use either with liquid products or mixtures of solid products and brine. The use of the method requires the premise that every sample removed represents an adequate and representative sample of the contents of the tank at the time of sampling. The use of the tank with viscous products such as cream style corn, purees, puddings, and condensed soups would appear to offer more problems than with products containing thin liquids. Samples removed from the tank may be used for determination of the number of viable spores remaining or subcultured either into media or tubes of sterile product for end point determination.

In the case of heating in phosphate buffer or other non-nutrient reference substrate, subculture of the heated suspension is necessary. If heating is conducted on spores suspended in a food product or other nutrient medium, survival may be determined either by subculture or by product incubation. The indicated survival time may vary markedly depending upon whether subculture or product incubation is used. With products of somewhat acid pH (4.3 to 5.5) it appears in general that subculture may indicate much longer survival times than product incubation (Sognefest *et al.*, 1948; Reed, Bohrer and Cameron, 1951; Reynolds *et al.*, 1952). While pH may be the main factor involved in inhibiting the germination and development of heated spores in food products, other factors of an inhibitory nature may play a part, as also may the nutritional adequacy of the particular product in relation to the requirements of the surviving organism.

The determination of the presence of surviving organisms by subculture is also attended by other factors introducing difficulties and uncertainties. Curran and Evans (1937) pointed out that spores of certain organisms and also cells of *Escherichia coli* which survived heat treatment or irradiation appeared to be more exacting in their growth requirements than before treatment. Enrichment substances such as glucose, blood, and milk added to nutrient agar gave appreciably higher numbers of survivors than nutrient agar alone. Nelson (1943, 1944) also has found that the number of survivors obtained is dependent upon the composition and nutrient value of the recovery medium. In addition to the possible enhanced nutrient requirement it has also been shown that heating may increase susceptibility to inhibitory substances present in media. Olsen and Scott (1946) found media to contain substances which inhibited the germination of spores of *Cl. botulinum*. The inhibitory effect could be overcome by the inclusion of starch in the medium.

The inhibitory effect was marked for spores which had survived heat treatment and almost negligible for unheated spores. Wynne and Foster (1948a) confirmed the effect of starch on removing inhibitory substances preventing the germination of mildly heated (176° F 20 minutes) spores of *Cl. botulinum*. Foster and Wynne (1948a) have shown long chain unsaturated fatty acids to have an inhibitory effect on spore germination which could be prevented by the incorporation of starch. Halvorson (1950) has suggested that fatty acid peroxides rather than the fatty acids are the inhibitory factor, and Roth and Halvorson (1952) have shown considerably greater inhibitory activity by oxidized fats or fatty acids on the germination of spores of several species as tested by colony development in media than was obtained with unoxidized fats or fatty acids.

Olsen and Scott (1950) made a detailed study of the inhibitory effects of media upon the germination of spores of *Cl. botulinum* and other clostridia. Starch, charcoal and serum albumen were shown to overcome the effect of substances naturally present in the culture media used which prevent the germination of some surviving and potentially viable spores. Similar behavior and effects were shown to occur for members of the *Bacillus* group by Murrell, Olsen and Scott (1950). As the period of heating was increased the surviving spores appeared to become increasingly sensitive to the inhibitors present in the medium. Starch, charcoal and serum albumen act to remove the inhibitory effect in a manner similar to that found with the clostridia. These authors conclude that the experiments do not furnish evidence regarding the nature of the inhibitory substance although long chain unsaturated fatty acids may be involved.

It is evident that the determination of survivors or of endpoint of survival from heat sterilization tests is no simple matter and that the results obtained may depend to a large degree upon the particular substrates used for determining survival.

Both time and temperature of incubation may affect the results obtained for recovery cultures of spores surviving heat resistance tests. Williams and Reed (1942) found that lower temperatures of incubation (75° - 85° - 87° F) gave higher apparent resistance than 98° F for spores of *Cl. botulinum* surviving heating tests. Similar results were obtained for PA 3679, a clostridium used extensively in the canning industry as a test organism. Much unpublished work in several laboratories has confirmed this result, so that at present most laboratories concerned with the canning industry have adopted incubation temperatures of 80° to 85° F for work with the mesophilic clostridia. Sugiyama (1951) has recently published

results on the effect of incubation temperature for *Cl. botulinum* in agreement with those of Williams and Reed. Incubation temperatures for spore recovery of members of the genus *Bacillus*, however, have not been so well established. In the case of the obligate members of the group, temperatures of 120° to 130° F appear to be standard. For the mesophilic or facultative members temperatures of 85° to 98° F appear to be used, depending upon preferences of the individual worker. There have been no studies reported upon the effect of incubation temperature upon apparent thermal resistance similar to those on the clostridia. There are some indications that incubation temperatures used for members of the *Bacillus* group may be at least as important as with the clostridia in determining the results obtained with recovery cultures obtained from heated spores. This is a problem which deserves and requires extensive investigation if reliable information on the thermal resistance of these organisms is to be obtained. There seems to be little or no information with respect to possible effects of incubation temperature upon survival results obtained with non-sporeforming organisms. In most cases recovery cultures have been incubated at temperatures considered optimum for the normal growth of the organism concerned.

The time of incubation of recovery cultures either for the enumeration of survivors or for qualitative survival is also important. Marked dormancy or delayed germination of heated spores of *Cl. botulinum* was reported by Esty and Meyer (1922) and Dickson (1928) and others. Dormancy in some cases may be associated with the culture environment (Morrison and Rettger, 1930a, 1930b; Wynne and Foster, 1948a) and in some cases may be reduced or eliminated by provision of better nutrient conditions or elimination of inhibitory substances. However, in the case of product incubation tests or in subculture tests where all nutrient factors or potential inhibitors are not known, delayed germination is a very general fact which must be taken into consideration. The proper incubation time is a rather arbitrary decision which must be established by each worker on the basis of experience. In general the attitude should be taken to make incubation times as long as practical when there is no previous experience as a guide. In this laboratory product incubation in thermal death time cans with mesophilic clostridia is continued for at least 120 days and samples are held longer if practical. It is quite common to observe spoilage to occur after 75 to 100 days incubation, and in some cases swells have occurred after 200 days incubation. Product incubation for thermophilic anaerobes is continued for 60 days although experience has indicated no spoilage after 30 days incubation at 130° F. Product incubation tests



in thermal death time cans are continued for three weeks with the members of the *Bacillus* group. This arbitrary time has been selected pending further study and in view of some results indicating shorter incubation periods as insufficient. The length of incubation time which has been used, as reported in the literature, is very variable and in some cases has not been specified when thermal resistance results are reported. In two publications in recent years, which will not be cited specifically, thermal death time data have been reported in which the authors failed to give either the incubation temperature or incubation time on which the results were based. In closing this portion of the discussion it may be said that germination of surviving spores or growth of vegetative cells surviving heat treatment is a complex function of time and temperature of incubation and of nutrient and inhibitory factors. Together and interrelated they have an important bearing on when a cell is "dead."

All determinations of thermal resistance are based on some initial number of cells or spores. In the case of vegetative organisms the determination of initial number does not seem to have presented any problem, or at least none has been recognized, as enumeration is carried out on whatever is considered an adequate medium from a nutritional standpoint. Determinations of the number of spores originally present in a suspension are plagued by the effects of some of the factors previously mentioned and also some specifically new. Inhibitory substances naturally present appear to be less active against unheated spores (Olsen and Scott, 1950; Murrell, Olsen and Scott, 1950) although added fatty acids are inhibitory to mildly heated spores (Foster and Wynne, 1948a). Length of incubation and incubation temperature may not be of as great importance in many cases, although there are few studies which answer this problem. Williams and Reed (1942) found higher counts at lower incubation temperatures with *Cl. botulinum* spores mildly heated (218° to 228° F). Curran and Evans (1945) found that mild heating (205° F, 10 minutes) caused heat activation of spores of many organisms so that spore counts were higher on a suspension following heat treatment than on the unheated suspension. The degree of "activation" was dependent upon the suspending medium in which heating occurred and also upon the temperature of incubation. Since most spore suspensions are mildly heated to eliminate vegetative cells in order to obtain spore counts, some degree of activation is probably obtained in all spore counting procedures. The activation of spores by mild heating has been reported for spores of PA 3679, Reynolds and Lichtenstein (1949) and Stumbo, Murphy and Cochrane (1950). The exact degree of heat activation and

its dependence upon time and temperature of heating as well as upon the suspending medium is probably a variable characteristic of each particular suspension and must be taken into consideration whenever viable spore counts are made.

Wynne and Foster (1948b) found that small amounts of carbon dioxide were required for the germination of spores of *Cl. botulinum* in a synthetic medium, and the absence of carbon dioxide very markedly delayed the germination of spores in a complex organic medium. Four other anaerobic organisms including PA 3679 were not affected by the exclusion of CO<sub>2</sub>. It is thought that these organisms can use certain substances in the complex organic medium to by-pass the CO<sub>2</sub> requirement, whereas *Cl. botulinum* cannot. This would be in accord with results with other organisms (Lwoff and Monod, 1947; Aji and Werkman, 1949). Andersen (1951) has reported that the addition of NaHCO<sub>3</sub> (0.10 to 0.15 per cent) to media for counting spores of *Cl. botulinum* was essential for prompt and uniform development of colonies and for maximum counts to be obtained. Substantial confirmation of the CO<sub>2</sub> effect on viable spore count of a suspension of *Cl. botulinum* 62A has been obtained by other laboratories (Olcott, 1952). The writer has had the opportunity to confirm the effect of added NaHCO<sub>3</sub> on the spore count of *Cl. botulinum* and found that the effect of added NaHCO<sub>3</sub> is also interrelated with the composition of the medium and may be quantitatively different in different media. Reynolds *et al.* (1952) reported higher spore counts of PA 3679 when NaHCO<sub>3</sub> was added to the medium. These results all point to the necessity for much more investigation of the conditions governing the germination of spores with respect either to the determination of initial spore counts or the detection of survivors of a thermal resistance test.

## FACTORS AFFECTING THERMAL RESISTANCE

It is very difficult to make a just appraisal of the factors affecting thermal resistance from any survey of the literature. The many variables introduced by the type of organism studied, the techniques used, and the unrecognized involvement of the many factors previously discussed, make a sound interpretation of previously reported data very difficult. The writer feels that a detailed critical analytical review of past work in this field which would evaluate the conclusions reached by individual authors in the light of the techniques used, and attempt to sort out in useable form only that work which is not invalidated or rendered questionable by techniques or procedures later shown to be faulty, is a necessary foundation to the most productive future development of this field

of study. However, under present limitations, the writer must follow the accepted procedure of citing the results and conclusions as given in the literature in an attempt to portray to some degree the effect of the various factors which may influence the thermal resistance of microorganisms.

There may be three general types of factors which affect thermal resistance:

- a) inherent resistance.
- b) environmental influences active during the growth and formation of cells or spores.
- c) environmental influences active during the time of heating of the cells or spores.

Inherent resistance is illustrated by the fact that in the same medium and under the same growth conditions different strains of the same species or same general type of organism may produce cell or spore suspensions having widely different degrees of resistance. This is shown for obligate thermophilic flat sour types (Bigelow and Esty, 1920) *Cl. botulinum*; (Esty and Meyer, 1922; Esty, 1923; Dickson *et al.*, 1925). A similar type of behavior has been shown for vegetative cells of bacteria (Beamer and Tanner, 1939a) and yeasts (Beamer and Tanner, 1939b).

Environmental influences active during the formation of cells or spores are illustrated by the effects of age, incubation temperature, and composition of the nutrient medium considered in relation to any one particular organism.

Environmental influences active during the heating of the suspension comprise all those variables such as pH, carbohydrate, protein and fat content of the substrate, colloids such as starch or soil, salt, and many other soluble organic or inorganic compounds which might be present.

Since what may appear to be inherent differences in resistance, when several cultures are compared on one arbitrarily selected medium, may develop into the effects of environmental influences if the same cultures are studied in relation to some selected environmental variable, and the relative resistances of two organisms appearing in one suspending medium such as phosphate buffer may be either accentuated or minimized by suspending in another substrate, all comparisons and evaluations become difficult and elusive as also do the previously attempted distinctions.

*Age.*—Esty and Meyer (1922) found young moist spores more resistant than old moist spores. Magoon (1926b) reported complex interrelationships between length of storage, storage temperature, and humidity upon the resistance of spores of *Bacillus mycoides*. It should be mentioned that the total range of time of survival



within which differences were regarded as significant was 3 to 7 minutes at 212° F. Sommer (1930) found the maximum resistance of spores of *Cl. botulinum* to appear in 4 to 8 days. Curran (1934) found ageing up to one year slightly increased resistance. However, Williams (1936) could find no correlation between age and resistance of spores of several different species of spore forming organisms. Further references to the problem of age and resistance may be found in the publications cited. There appears to be no possibility of reaching any general conclusion with regard to the effect of ageing upon the resistance of bacterial spores based upon the data available in the literature. Furthermore, it may be questioned whether much of the data reported were adequate to measure "age," howsoever defined as the single variable factor.

*Growth Phase.*—It has been generally observed that vegetative bacterial cells show differing degrees of susceptibility to adverse influences at various stages of the growth cycle (Sherman and Albus, 1923; Sherman and Cameron, 1934). Robertson (1928) and Stark and Stark (1929) found young cells more susceptible to heat than older and more mature cells. Anderson and Meanwell (1936) found increased resistance of thermoduric streptococci during the early logarithmic phase, while Ellicker and Frazier (1938) working with *E. coli* found greater heat resistance during the initial stationary phase with decreasing resistance when reproduction began and a minimum resistance during the period of most rapid reproduction. There is sufficient evidence in these and other publications to demonstrate that studies of the resistance of non-sporeforming organisms must take into consideration the factors of growth phase and "age."

*Temperature.*—The effect of growth temperature has been studied rather extensively. Weil (1899) found the resistance of spores of *Bacillus anthracis* to increase with growth temperature. Sames (1900) studied the resistance of spores of thermophilic members of the *Bacillus* group and found that spores produced at higher temperatures are more resistant than spores produced at lower temperatures. Williams (1929), Curran (1934), and Sobernheim and Mündel (1936) have also found resistance of spores to increase with incubation temperature. Theophilus and Hammer (1938) found that the spores of cultures from evaporated milk showed maximum resistance at the optimum growth temperature. Several subcultures at a given temperature were necessary before the maximum effect of temperature became apparent. Lamanna (1942) presented a general relationship between maximum growth temperature and spore resistance of various members of the *Bacillus* group which, however, only included organisms of comparatively

low resistance, not over 45 minutes at 212° F. Sugiyama (1951) reported higher resistance of spores of *Cl. botulinum* produced at higher temperatures than those produced at lower temperatures.

*Nutrient Conditions.*—Williams (1929) has reviewed the earlier literature and conducted extensive studies of the effect of nutrient conditions and other factors on the resistance of spores of a strain of *Bacillus subtilis*. Many of the nutrient conditions tested either increased or decreased the resistance compared to one nutrient condition taken as standard. Different brands of peptone may result in differing resistance, although resistance appeared to be independent of concentration with any one peptone. Various digest mediums resulted in spores of low resistance except for Casein digest which enhanced resistance. Spores of high resistance were found in all media prepared from vegetable extracts and also in isoelectric gelatin. The addition of either phosphate or magnesium to the standard peptone medium increased resistance. The addition of available carbohydrates, organic acids or amino acids in some cases increased resistance. It is very interesting to note that the increases in resistance obtained with varying nutrient conditions were reflected only in the spores as thus produced and transfer to the standard test medium restored the original resistance. Therefore the increased resistance produced by nutrient conditions appeared to be solely the effect upon the spore that was formed and not to be transmissible. Curran (1935) found that spores produced and aged on soil or oats were more resistant than those from artificial media, whereas Görtzen (1937) concluded that there was no significant difference in the resistance of spores of anaerobic organisms taken directly from soil and those from cultures, except for one organism, *Cl. sphenoides*. Sobernheim and Mündel (1938) found that soil containing spores required 6 to 8 times as long to sterilize as a similar number of spores cultured from soil. Gillespy (1947) working with thermophilic anaerobes found an appreciable increase in resistance of the spores when soil was included in the medium. Sugiyama (1951) using *Cl. botulinum* found the resistance of the spores to be influenced by the composition of the culture medium. Reduction of iron or calcium concentration decreased resistance. The inclusion of fatty acids tended to increase resistance, notably with the long chain unsaturated fatty acids. Extraction of the medium with chloroform or petroleum ether reduced the resistance of the spores produced on the extracted medium.

*pH.*—In general the greatest thermal resistance is shown in a fairly broad pH zone centering on the neutral point  $\text{pH} = 7$ . Although there are variations due to the nature of individual

organisms, resistance is at the maximum in the pH range 6.0 to 8.0. This zone is somewhat broader or narrower depending upon the particular suspension, the suspending medium whether buffer of food substrate, the technique used in the determination, and the subculture conditions used (Bigelow and Esty, 1920; Esty and Meyer, 1922; Williams, 1929; Lang, 1935; Nichols, 1940; Gillespy, 1948) and many others. Sognefest *et al.*, (1948) studying the resistance of spores of *Cl. botulinum* and PA 3679 heated and incubated in thermal death time cans in pureed food products adjusted to various pH levels, concluded that in the range 4.5 to 9 the lower the pH, the lower the heating required to prevent spoilage. Between pH 6 and pH 9 the change in resistance was comparatively minor, in the region of pH 5.5 and below resistance fell off rather sharply. In this case resistance is measured both by killing and the ability of any surviving spores to germinate in the product at that particular pH. Somewhat different results in relation to pH may be found if after heating in the acid pH range the survivors are subcultured into a neutral medium. The effects of pH on thermal resistance and spore germination in food products are very complex since pH changes not only may have an effect directly upon the organism, but also by altering the degree of dissociation of many substances in solution in the product or by producing a shift in the oxidation-reduction potential may indirectly exert effects either upon survival or upon the ability of surviving organisms to develop. In general it would appear that, for spores at least, the subculture of suspensions heated in food products below pH 5.5 to 6.0 to more neutral media will lead to longer indicated survival times (Sognefest *et al.*, 1948; Reed, Bohrer and Cameron, 1951; Reynolds *et al.*, 1952).

Aref and Cruess (1934) found yeasts to be slightly more resistant at pH 3.8 to 4.0 than at a more neutral reaction, while Beamer and Tanner (1939*b*) found all but one of the yeasts tested to have greater resistance at pH 6.8 than at pH 3.8. Gillespy (1936-37) reported the ascospores of *Byssochlamys fulva* to have a maximum resistance at pH 5.0, with resistance greater at 3.0 than at 7.0. Among other studies on the effect of pH upon the thermal resistance of non-sporeforming organisms are those of Levine and Fellers (1940) and Skillington and Levine (1943). Further references to the extensive literature on this subject may be found in most of the papers cited.

*Carbohydrates.*—High concentrations of soluble carbohydrates quite generally result in an increase in resistance of yeasts (Peterson, Levine and Buchanan, 1927; Wallace and Tanner, 1931); non-sporeforming bacteria (Fay, 1934; Baumgartner and Wallace,



1934; Baumgartner, 1938); and bacterial spores, (Weiss, 1921a; Rahn, 1928; Von Angerer and Kuster, 1939; Braun, Hays and Benjamin, 1941; Anderson, Esselen and Fellers, 1949; and Sugiyama, 1951). Although the magnitude of the effect is great, in some cases resulting in increases of resistance of 200 to 300 per cent, the mechanism is obscure. The increase in resistance has been attributed to partial dehydration of the protoplasm. Fay (1934), Von Angerer and Küster (1939) and Sugiyama (1951) have observed marked decreases in turbidity or increases in light transmission for cells or spores suspended in carbohydrate solutions. Rahn (1945b) and Sugiyama (1951) do not believe the theory of dehydration is sufficient to fit all the facts, since protection is not proportional to molarity.

The very marked and general effect of soluble carbohydrates in increasing the thermal resistance of almost all organisms tested offers a point of approach to the study of the mechanisms of thermal destruction which should be exploited more fully by further investigation.

*Salt.*—Weiss (1921a) found 3 per cent salt to decrease the resistance of *Cl. botulinum* spores. Esty and Meyer (1922), however, found an increase in resistance with 1 to 2 per cent salt and a decrease in resistance above 8 per cent. Viljoen (1926), working with spores of thermophilic flat sour organisms, found resistance in a pea liquor, prepared by processing peas with distilled water, to be increased by the addition of salt. Increasing amounts up to 3 per cent increased resistance. The addition of 0.25 per cent salt to water decreased the resistance of the same organisms. It is apparent therefore that the protective effect of salt in this case is related to some constituent of the pea liquor. In view of the striking results reported by Viljoen it is somewhat surprising that further work upon these lines has not been conducted.

Headlee (1931) found 2.5 per cent salt to increase the resistance of spores of *Cl. welchii* while 10 per cent led to a definite decrease. Yesair and Cameron (1942) found 3.5 per cent salt in phosphate buffer to decrease the resistance of spores of *Cl. botulinum* at temperatures below 230° F, but there was no appreciable effect at 230° to 235° F. Anderson, Esselen and Fellers (1949) added 1, 2, 4 and 8 per cent salt to tomato juice and found increasing salt concentrations to decrease the destruction time of spores of *Bacillus coagulans* (*Bacillus thermoacidurans*). Sugiyama (1951) obtained an increase in resistance on the addition of 0.15M (0.8 per cent) salt to phosphate buffer. Apparently the effect of sodium chloride and perhaps of other salts is variable depending upon the suspending medium and the particular test organism used.

The role of sodium chloride and other meat curing salts upon the thermal resistance of spores in relation to meat processing has been discussed by Yesair and Cameron (1942), Jensen (1945), Stumbo *et al.*, (1945*b*, 1945*c*), and Bulman and Ayers (1952).

*Fats.*—Lang and Dean (1934) found increased survival and resistance in spores of *Cl. botulinum* in fish products in oil. Jensen (1945) reported increased resistance of streptococci in fatty as compared to aqueous media. Yesair, Bohrer and Cameron (1946) found that dried cocci heated in moist fat had considerably greater resistance than the same cocci resuspended in broth. Suspended in dry fat the organism showed a very pronounced increase in resistance. They believe that the mechanism of increased resistance is based upon the localized absence of moisture. Much more work needs to be done upon resistance in fats or fatty emulsions in order to clarify the results sometimes obtained in adequately processed products which yield viable organisms.

*Antibiotics.*—The study of the effect of antibiotics on thermal resistance was stimulated by the report of Andersen and Michener (1950) of the successful use of subtilin and mild heat to preserve a variety of foods. Subsequent investigations by a number of workers have failed to confirm these results (Bohrer, 1951; Burroughs and Wheaton, 1951; Adams, Ayers and Tischer, 1951; Williams and Campbell, 1951; Cameron and Bohrer, 1951; Williams and Fleming, 1952). The main conclusion reached has been that the action is sporostatic rather than lethal, and in most cases of a transient nature. Le Blanc, Devlin and Stumbo (1953), however, have reported that small quantities of subtilin (14 ppm) in pea puree reduced the thermal resistance of spores of PA 3679 47 per cent and of *Cl. botulinum* 63 per cent. Since subculturing of the heated suspensions diluted the subtilin out beyond inhibitory activity it appears that the presence of the antibiotic in a complex medium was capable of reducing the thermal resistance.

There may be many substances of comparatively simple chemical structure which may either increase or decrease the thermal resistance of microorganisms. Almost any organic chemical compound might be included in this class and its presence in a given complex substrate be primarily responsible for the particular level of thermal resistance which occurs. Continued investigation of the effects of chemicals of known nature and composition, added to simple and complex substrates, upon the thermal resistance of microorganisms will undoubtedly contribute considerably to both the theoretical and practical aspects of sterilization by heat.

## RESISTANCE TO DRY HEAT

The results of many experiments on vegetative cells and spores of bacteria and upon yeasts have demonstrated without doubt that organisms are much more resistant to dry heat than to moist heat. Rahn (1932, 1945b) has summarized and discussed some of the literature. In general most of the methods used for measuring resistance to dry heat in the temperature range above 212° F have not had a very high degree of precision and most data for exposure time include an uncorrected lag time which may be variable depending upon the technique used and in many cases might represent a considerable proportion of the total exposure time. In spite of this, however, the vast difference in time for destruction by moist heat and dry heat might be illustrated by the data of Tanner and Dack (1922) on spores of *Cl. botulinum*; while the maximum moist heat resistance of these spores is 2.78 minutes at 250° F in phosphate buffer (Esty and Meyer, 1922; Townsend, Esty and Baselt, 1938), the following dry heat survival times were found in different experiments; 284° F, 15 to 60 minutes; 302° F, 10 to 25 minutes; 230° F, 10 to 15 minutes. The destruction times were five minutes longer in each case than the figures cited. The slope or  $z$  of the destruction curves as approximated from the results on many organisms is of the order of 40 - 75, with the consequence that starting from any reference point considerable increase in temperature must take place to appreciably reduce destruction time.

The possibilities of the commercial exploitation of aseptic canning procedures in which cans and covers are sterilized by super heated steam or other hot gases (Martin 1948, 1950) have renewed interest in the study of resistance to dry heat. Investigations are in progress in several laboratories and it may be expected data on dry heat resistance of many organisms secured by improved and more precise methods will become available.

## MECHANISM OF RESISTANCE AND THE CAUSES OF DEATH BY HEAT

The physical and chemical conditions existing within the cell or spore of a microorganism which are responsible for the much greater resistance to heat of these forms of life than of any other are exceedingly obscure. The processes occurring within the cell which under any given set of conditions lead to the loss of viability and reproductive capacity, which by definition constitutes the death of a microorganism, are of great complexity and undoubtedly involve differing mechanisms, depending upon the nature of the lethal agent.



With specific reference to death by heat the data and theories existing in this field have been reviewed by Williams (1929), Rahn (1932, 1945*b*), Curran (1952) and discussed by many others. Rahn (1945*b*) states without qualification that, "Death by dry heat is due primarily to an oxidation process; death by moist heat is due to coagulation of some protein in the cell." In view of the enormous variation in thermal resistance displayed by vegetative cells and more particularly by bacterial spores, the marked effect of environmental conditions on the resistance of some organisms and the relative refractoriness of others to environmental effects, and the absence of any really crucial experiments designed to investigate the mechanisms of resistance or the causes of death, it appears to be rather premature to hold too solidly to any particular explanatory hypothesis.

The high temperature coefficient for death by moist heat gives some evidence that a denaturation or coagulation process is involved. However, the marked effects which subculture media and conditions may have upon the development of survivors suggest that heat damage leading to loss of viability must be a more complicated process than that of inactivation of some essential molecule by denaturation or coagulation. Autosterilization, or loss of viability of some heated spores when held below growth temperature (Pearce and Wheaton, 1952), suggests that heat damage is not always immediate and final, but that as a result of heating further changes in an organism can take place which lead to death. No further attempt will be made to discuss and evaluate the large mass of existing data on thermal resistance to reach any conclusions as to mechanisms. Much more work is desirable in this field, particularly experiments designed to test specific theories of the causes of resistance or the mechanism of death.

### RESISTANCE OF SURVIVORS

The resistance of new suspensions prepared from the survivors of thermal treatment has both practical and theoretical significance. Practically, if it occurs, it might be responsible for gradually increasing levels of resistance in any naturally occurring population; also it may make possible the selection of organisms for test purposes with desired levels of resistance. Theoretically it has significance in relation to the nature of survivor curves and mechanism of death. The existing evidence is contradictory. Bigelow and Esty (1920), Magoon (1926*a*), Williams (1929), and Davis and Williams (1948) have by means of selection of survivors obtained cultures having significantly higher resistance than the original. Morrison and Rettger (1930*a*), Sommer (1930), Williams (1936),

and Desrosier and Esselen (1951) have been unsuccessful in obtaining populations of higher resistance by the cultivation of survivors.

While some of the data may be criticized justifiably (Rahn, 1932), increase of resistance by selection in some cases appears to be a fact. From the contradictory nature of the data it seems probable that it may occur with one organism and not another, or under one set of cultivation conditions and not another. In view of the practical and theoretical implications involved, continued study of this problem with a variety of organisms and techniques is very desirable.

It is readily apparent from this review that there are a large number of factors involved in evaluating the thermal resistance of microorganisms. The search for and investigation of those factors and conditions which may influence the thermal resistance of microorganisms would seem an unending task, but one which is assuredly profitable either in providing explanatory data for phenomena previously observed or serving to point to means for more adequate utilization of heat for the practical purposes of sterilization.

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## 33

# COMMERCIAL PROCESSING OF CANNED FOODS

COMMERCIAL processing of canned foods by the application of heat is designed to protect the health of the consumer and to preserve the product from microbiological spoilage which could result in economic loss. The basic or minimum requirements for the commercial processing of canned food have been expressed in various ways. Ball (1929) states "For a food to be made commercially sterile, all pathogenic organisms and all other organisms which can grow at ordinary storage temperatures, must be destroyed. Perhaps the most resistant as well as the most dangerous pathogenic organism which may be present in canned food is *Bacillus botulinus*." Bulletin 26L of the National Canners Association (1950) states "The term 'process' as used in this bulletin designates the heat treatment expressed in terms of temperature and time given the product after the container is permanently sealed. The basic, or minimum, requirement for the process for any product is that it be sufficient to destroy the organism of greatest known resistance to heat, which, by reason of its survival may be detrimental to health."

In general most processes which meet the basic or minimum requirement from the standpoint of public health are not adequate from the standpoint of preventing spoilage by more heat resistant organisms which would result in serious economic loss. Therefore the major problems in establishing processes have been concerned with protection against potential spoilage organisms of resistance higher than that of any organisms significant from a health standpoint.

In addition to the consideration of the minimum requirements for preservation there is also the factor of the maximum possible retention of quality, palatability and nutritive value. Commercial processes today represent the best balance which is compatible



with adequate preservation against spoilage and the maximum retention of quality based upon present knowledge of the factors involved in process determination and calculation.

The principles underlying the determination of commercial processes will be discussed first in relation to the processing of hermetically sealed containers in still retorts since it was for these conditions that the elements of commercial processing were first established and most of the newer and different methods of processing depend upon an extension of the same basic principles.

Although all commercial processes in still retorts are expressed as a time and a temperature, the basic consideration is the lethality or sterilizing value of the process achieved within the container by the specified time and temperature. For any given retort temperature and process time the lethality or actual sterilizing value is a function of the can size and the heating characteristics of the product.

Although early measurements were made of the temperatures achieved inside a can by the use of maximum temperature reading thermometers (Ball, 1938), later more useful data were obtained by means of thermocouple measurements which permitted the determination of the rate of heat penetration at any point within the container and the temperature distribution within the container at any given time (Bitting and Bitting, 1917; Thompson, 1919; Bigelow *et al.*, 1920). Ecklund (1949) developed a non-projecting type thermocouple which greatly facilitates the measurements of heat penetration and is in rather extensive use today. Following the development of methods of measuring satisfactorily the heat penetration and heating characteristics of canned foods the methods for the evaluation of heat penetration data were developed. Bigelow *et al.* (1920) used a graphical procedure, since then known as the General Method, in order to obtain the summation of the lethal effect at the measured point in the container. Ball (1923, 1928) introduced the mathematical methods for thermal process evaluation. This included the definition of factors to characterize the heat penetration curve and the formulae for the mathematical integration of the lethal effects produced by time-temperature relationships at the point of greatest temperature lag within the container. In order to have a uniform basis of expression Ball (1928) introduced the symbol  $F$  to designate the time in minutes required to destroy an organism at 250° F. Using the heat penetration factors as defined and the mathematical method of integration, the data of any heat penetration test can be calculated to equivalent minutes at 250° F. When this is calculated assuming  $z = 18$  for the slope of the thermal death time curve, the resultant value is given the

symbol  $F_0$ . In general heat penetration data are calculated for a slope of 18 and results expressed as  $F_0$  for the sterilizing value of the process. Methods of process evaluation have received further consideration by Schultz and Olson (1938, 1940), Olson and Stevens (1939), and Jackson and Olson (1940). The general theory of heating curves and heat transfer have been considered by Olson and Jackson (1942) and Olson and Schultz (1942). No attempt has been made here to present in detail the procedures for the evaluation of heat penetration data and the calculation of the sterilizing value of a process, which is considered beyond the scope of the present contribution. Ball (1943, 1948) and Stumbo (1949b) have reviewed and presented thoroughly the essential features of the graphical and mathematical methods of process evaluation based upon heat penetration data. Alstrand and Ecklund (1952) have recently reviewed the procedures for the measurement and interpretation of heat penetration of canned foods.

Stumbo (1948, 1949a, 1949b), Gillespy (1951), and Hicks (1951, 1952) have suggested new approaches to the evaluation of process lethality which differ from the conventional methods at present in use. According to Hicks (1952) "The newer ideas have provided a logically satisfactory basis for process evaluation for one which was logically quite unsatisfactory. This is an important gain from the academic point of view. Further, this work should facilitate clear thinking about the criteria to be adopted and the relative importance of various factors affecting process values. This is likely to prove its most important practical consequence." Alstrand and Ecklund (1952) remark in reference to this new approach "All of these workers have approached the problem from the premise that there is probability of survival of spoilage bacteria throughout the can contents and not just at the can center. While the development of these methods will be followed with great interest by students of processing technology, there is but little likelihood that greatly different results will be obtained by application of the new methods as compared to the methods in common use." Further judgment of the value of these new concepts of process evaluation must await the results achieved by their application to practical processing problems.

Assuming that adequate heat penetration tests have been made it is then possible to calculate the sterilizing value for any given time temperature combination, where sterilizing value is expressed as  $F_0$ , that is the equivalent minutes at 250° F. A process for the product can then be selected to give the desired  $F_0$  value. The selection of  $F_0$  value of the process for a new product may be a somewhat complicated matter, which takes into consideration any

available thermal resistance data upon the types of organisms expected to be present, some estimate of the level of contamination, similarities to other products in composition and pH and the effect of proposed processing levels upon the quality of the product, always with the primary consideration that the lethality of the process *must* exceed the maximum known resistance of the spores of *Cl. botulinum* if the pH of the product is greater than 4.5. As stated by Olson and Stevens (1939) "Selection of a proper  $F_0$  value for purposes of calculating a process requires a considerable amount of experience in processing studies and a thorough understanding of packing procedures as well as the nature of the product."

In many cases the validity of heat penetration data and process calculation may be checked by inoculated packs. This procedure has been described by Meyer (1931), Lang (1935), Cameron (1936), and Williams (1940). In this procedure the thermal resistance of suspensions of spoilage organisms is first determined in the laboratory, preferably in the product under consideration. Then under commercial packing conditions containers of the product are inoculated with known amounts of spores and processed to give different sterilizing values based upon the accepted heat penetration data. As suggested by Williams (1940) several process levels should be used so that if possible 100 per cent spoilage should occur at one level, partial spoilage at the next higher level and no spoilage at the next higher level. If no spoilage occurs at the process level which is expected to produce sterility based upon the laboratory thermal resistance data it may be concluded that the inoculated pack has justified the validity of the heat penetration measurements and process calculations. The previous remarks relate to the inoculated pack in theory. In actual practice the inoculated packs depend to a great degree upon the adequacy of standardization of the suspensions. Standardization of suspensions for inoculated packs requires attention to the many considerations which have been discussed previously (Chapter 32) in relation to the determination of thermal resistance. Adequate and satisfactory inoculated packs can be obtained only when the laboratory data are sufficient to thoroughly establish the resistance of the suspension. If the slope of the thermal death time curve varies appreciably from  $z = 18$  in the product concerned it may be necessary to take this into consideration in calculating the sterilizing values of processes from the heat penetration data, particularly if the retort temperature is more than a few degrees above or below 250° F. Adequate distribution of the spore inoculum in the test containers, the inclusion of a sufficient number of containers at each process level and accurate control of the experimental processes are also required to give



satisfactory inoculated packs. It is now the generally accepted procedure to run heat penetration tests at the same time as the inoculated packs so that the process levels actually used in the inoculated pack work may be verified.

Although the largest proportion of commercially canned foods are processed in still retorts, other methods of processing by heat have been introduced. Some are in commercial operation, others are still undergoing experimentation and development. Ball (1938) very thoroughly reviewed the various procedures which have been proposed for the heat sterilization of canned foods. Jackson and Benjamin (1948) more recently have reviewed this subject with special reference to those methods which either were in commercial use or at an experimental stage which might promise early commercial development. Townsend (1952) has discussed several of the newer processing methods with special emphasis upon the control problems involved.

Continuous cookers of the rotary reel type would probably be second to the still retort in amount of canned foods commercially processed. These units are designed either for atmospheric or for pressure processes. The former is used extensively in processing acid products which do not require temperatures above 212° F, while the pressure type is used for evaporated milk, some formulated products and several low acid vegetable products. In addition to the advantages of continuous operation, the axial rotation of the can produced by passage of the cans through this type of cooker causes agitation of the product which results in increased rates of heat penetration. For any given temperature and process time therefore the sterilizing value is greater than for the same temperature and time in a still cook in a retort. The increased rate of heat penetration due to the agitation has made possible the use of higher temperatures and shorter process times for many products with resulting increase in quality of product. Since process requirements are based upon a sterilizing value or  $F_0$  of a process, considerable effort has been expended to measure the sterilizing values for continuous cooker processes at various temperatures and can speeds. Special equipment to reproduce the type of agitation while permitting the measurement of heat penetration has been constructed (Townsend, 1952), since it is not possible to measure heat penetration in a commercial unit. Havighorst (1953) has recently described the processing of cream style corn in one type of continuous cooker which illustrates the principles applied and the problems involved.

A radically different type of agitation for the purpose of increasing the rate of heat penetration into canned foods has been described by Clifcorn, Peterson, Boyd, and O'Neil (1950). The

cans are subjected to controlled end-over-end rotation around an axis external to the can in a special type of retort. Under the proper conditions of headspace, speed, and radius of rotation the rate of heat penetration is more rapid by this method than by any other known method of agitation. Marked improvements in quality were observed in many products with processes which exceeded in lethal value those usually obtained in still retort processes. The procedure appears to be particularly applicable to the processing of some viscous products in large size cans which cannot be processed satisfactorily in the still retort due to slow heat penetration and the adverse effects upon quality of the long processes required to obtain sterility. Further information upon the commercial exploitation of this principle is given by Conley, Kapp, and Schuhmann (1951) and by Anonymous (1953a) in relation to the canning of whole milk.

A number of acid products have been processed by heating, filling into the containers, sealing and holding for a sufficient length of time to sterilize the container. In general these products include fruit and tomato juices, pulps and pastes. The degree of prior heat treatment and the time of holding required for sterilizing the container depend upon the types of spoilage organisms to which the product is susceptible. In products sufficiently acid so that only yeasts and moulds and non-sporeforming bacteria are potential spoilage organisms, filling temperatures of the order of 160° to 170° F and a holding time of a few minutes appear to be sufficient. Tomato juice which is susceptible to spoilage by spore forming organisms, *Bacillus coagulans*, requires a boiling water process after a hot fill in order to produce a sterile product. Presterilization of the juice in continuous tubular or plate type heat exchangers at temperatures of 240° to 260° F followed by cooling to 190° to 200° F, filling sealing and holding for a time sufficient to sterilize the container prior to cooling has been extensively practiced in order to avoid the long boiling water processes and to assure sterility of the juice against the organisms of relatively high heat resistance which have been encountered in this product. The presterilization procedure has been discussed in detail by Sognefest and Jackson (1947). Other acid products sufficiently liquid to be pumped through heat exchangers may be processed in a similar manner providing the pH is such that the hot fill and holding time will sterilize the container.

Aseptic canning of the product into sterilized containers following presterilization of the product by passage through a heat exchanger serves to adapt the presterilization procedure to low acid products susceptible to spoilage by organisms which would

survive the hot fill and hold procedure used with acid products. Jackson and Benjamin (1948) describe one form of this procedure which involves filling and sealing the cans in an enclosed chamber in an atmosphere of live steam. The containers are sterilized under high pressure steam while low pressure steam is used to maintain a sterile atmosphere within the filling and sealing chamber.

A process whereby cans are sterilized in superheated steam or other hot gases and filled and closed in a chamber in an atmosphere of superheated steam or other hot gases has been described by Martin (1948, 1950). Any product sufficiently liquid to be satisfactorily pumped through a heat exchanger might conceivably be packed by this method. This procedure has been applied experimentally to a large number of food products (Martin, 1950), and is being used commercially on pea soup (La Bonte, 1951), and in several installations in the commercial processing of whole milk (Atkinson, 1951; Bloomberg and Hessey, 1951; Meyer, 1951; Anonymous, 1952). Bolanowski and Lineberry (1952) describe a heat exchanger of special design which will handle quite viscous products and report its experimental application to aseptic canning procedures. The use of this heat exchanger and the Martin aseptic canning system on cream style corn has recently been reported (Anonymous, 1953*b*). Townsend (1952) has discussed some of the problems involved in operation of aseptic canning equipment. Aseptic canning requires considerable technical knowledge regarding the possibilities of bacterial contamination occurring at any point in the equipment during the canning operation and rigid adherence to standards for resterilization of equipment whenever the operating procedure indicates that this is necessary. Container sterilization cycles must be adequately controlled and possess an ample safety factor. Product sterilization procedures in heat exchangers at temperatures of 270° to 300° F have very high theoretical lethality in terms of  $F_{90}$ . The lethality of such processes must be checked and shown to be adequate by inoculated pack tests with spore suspensions of representative spoilage organisms of known resistance. Accurate control of product sterilizer temperature is important in this high temperature range, since variation of a few degrees in temperature makes a considerable difference in lethality in terms of  $F_{90}$ .

Another new development in canned food processing is the Smith-Ball procedure (Townsend, 1952), in which the product is presterilized by any suitable short time high temperature process and filled into cans at the processing temperature in a pressurized room. The cans are held for a sufficient time to complete sterilization, then partially cooled to prevent can distortion, and removed from the room for the completion of cooling at atmospheric pres-



sure. The empty cans and covers may either be completely pre-sterilized or partially sterilized prior to the high temperature filling operation.

The successful commercial processing of canned foods requires careful attention to several other factors in addition to the application of proper time and temperature of processing. Whatever the type of process equipment used there are problems of engineering control to assure efficient operation of the equipment. The problems herein involved are beyond the scope of this contribution. With reference to still retort operation they have been discussed in Bulletin 26L National Canners Association (1950) and Continental Can Company (1950) Research Bulletin No. 19. Equipment for the newer methods of processing which have been described are usually installed and started in operation under the specific direction of the manufacturer of the equipment.

Control of the bacterial load of the product to be processed is important. The established processes for canned foods are based upon a normal or average level of contamination with spoilage organisms. When the level of highly heat resistant organisms increases considerably, processes normally satisfactory may result in some spoilage. Therefore good sanitation and sanitary control are essential to the efficient and successful processing of canned food (Cameron, 1938; Cameron and Esty, 1940). All equipment used in the preparation and handling of the food product should be of the type which can be easily cleaned. Efficient cleaning programs to be applied during periods of shut-down must be established and controlled. The incoming raw products must be handled in a sanitary manner and with the view of removing as much contamination as possible. The problems concerned with sanitation are large and numerous and have received considerable attention from workers in the canning industry (Baumgartner, 1949).

In addition to the raw material which forms the bulk of the product processed, other ingredients such as starch, sugar, spices, flours and stabilizers are potential sources of introducing undesirable levels of contamination with heat resistant organisms. Bacteriological control of these ingredients by either the user or the supplier is essential. Considerable attention has been given to this phase of control in the canning industry (Yesair and Williams, 1942; Castell, 1944; Baumgartner, 1949).

Progress in the commercial processing of canned foods depends upon advancements in the basic sciences, chemistry, physics and microbiology, and upon engineering developments. Insofar as the best knowledge from these fields is adequately utilized continual improvement in the quality and stability of commercially processed canned foods may be expected.

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## 34

# STERILIZING BY IONIZING RADIATIONS

## INTRODUCTION

WITHIN a few years following the discovery of radium and x-rays, researchers in the biological sciences began to investigate the effects of these radiations on biological materials. Among the biological species studied to a great extent were microorganisms. Studies of the effects of ionizing radiations on microorganisms offer a great advantage over studies on higher biological species, in that it is possible to use large numbers of individuals in single experiments.

Although a great deal of early research was carried out by such pioneers in the field as Pacinotti and Porcelli (1898), Prescott (1904), and others, it was only as a result of the last war—with the impetus given to the field by development of high-energy particle accelerators capable of producing intense quantities of radiations—that considerable research was initiated to discover some practical utilization of the bactericidal effects of ionizing radiations.

The atomic era has made available immense concentrations of radiations produced both from particle accelerators (machine sources) and from isotopic sources, such as specific isotopes produced in piles at Oak Ridge and Brookhaven National Laboratories, as well as isotopes available in tremendous quantities as by-products of atomic fission.

A number of reasons make the use of ionizing radiations for sterilization of biological materials desirable. Among these are:

(1) Sterilization by ionizing radiations does not produce appreciable heat in the material being irradiated. A rise in product temperature of only about 4° C is caused by a sterilizing dose.

(2) Many food products, when heat processed, require more heat for sterilization than is desirable for optimal flavor.

(3) If it were possible to sterilize a product by ionizing radiations, the metal requirements for containers would probably be less than those at present needed for heat-processed products. Material of thinner gauge could be used, because the metal containers would not need to stand such rigid stresses as are put upon them in pressure processing. In addition, it would probably be possible to utilize more flexible films as containers for a number of products.

(4) Many heat-sensitive drugs and pharmaceuticals, which cannot now be sterilized by conventional heat methods, might be sterilized by ionizing radiations, which would result in a more uniform product, free of contamination.

It would appear highly desirable, therefore, that some attention be given to ascertaining whether ionizing radiations might be used for sterilization of biological materials such as foods, drugs, pharmaceuticals, and human tissues for subsequent transplant.

## FUNDAMENTAL ASPECTS OF ELECTROMAGNETIC AND PARTICLE RADIATIONS

To understand better the subject matter discussed later in this chapter, it is desirable to have a brief explanation of the various types of radiations that are available and a statement of the advantages and disadvantages of utilizing each type for sterilization purposes. It is not intended to give a complete description of each type of radiation but rather a brief summary of the important factors of each type as they affect its potential application for radiation sterilization.

### ELECTROMAGNETIC RADIATIONS

In general, radiations may be classified in two groups, (1) electromagnetic radiations and (2) particle radiations. Figure 57 presents a schematic diagram of the electromagnetic spectrum illustrating the approximate locations of various types of radiation. All the radiations to be found to the left of visible light, *e.g.*, radio waves, radar waves, and infrared rays, produce their bactericidal effects by means of heat produced in the material being treated. Those radiations in the electromagnetic spectrum to the right of visible light produce their bactericidal effects not by heat but by direct hits of the radiations on the target, (for instance, x-rays). This direct hit theory will be discussed in some detail later. It is these radiations, which produce their effects without heat, that one may consider for "cold sterilization."

*Ultraviolet Light.*—Ultraviolet light cannot be considered for sterilization of solid materials, such as foods in their final containers, because ultraviolet rays have but little penetration into matter and hence can accomplish only surface sterilization.

*X-rays.*—X-rays have relatively much greater penetration into matter. However, as only less than 5 per cent of the primary electron energy goes into the production of x-rays and the remainder produces heat in the target, the process of producing these radiations is extremely inefficient. In other words, x-rays, although sufficiently capable of penetrating the product, would require from

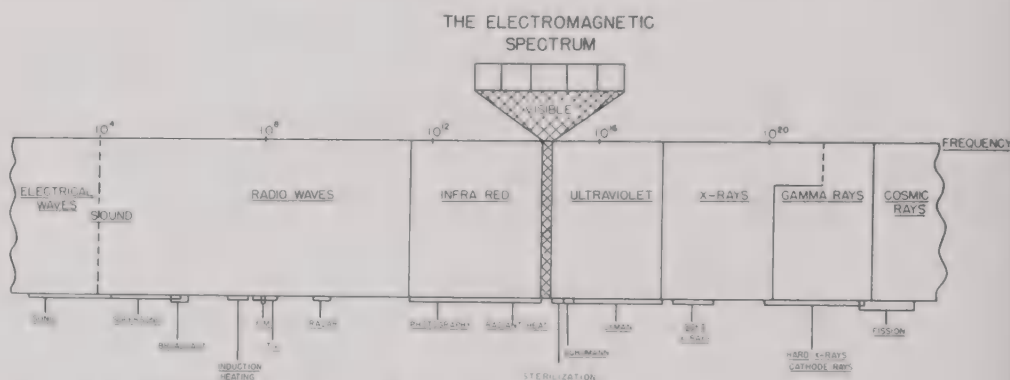


Fig. 57.—Schematic diagram illustrative of the electromagnetic spectrum, showing approximate locations of various types of radiation. (Proctor and Goldblith, 1951a)

10 to 20 minutes or more to sterilize even small quantities of a product. Therefore, from a practical standpoint, x-rays cannot be considered for this purpose.

*Gamma Rays.*—Gamma rays are high-energy radiations emitted from certain radioactive isotopes. With the advent of the Manhattan Project and subsequently the Atomic Energy Commission, large quantities of radioactive isotopes are potentially available as by-products of atomic fission. These isotopes are potential sources of a great amount of energy available as gamma radiation. These gamma rays have relatively great penetration into matter (as have x-rays) and are potential sources of radiation for sterilization purposes. These will be discussed in more detail later.

## PARTICLE RADIATIONS

*Cathode Rays.*—Cathode rays are artificially accelerated electrons or beta rays, *i.e.*, electrons accelerated by a man-made machine. Unlike x-rays, the efficiency of production of cathode rays is relatively high, for approximately 75 per cent of the energy of



the electron beam can be utilized. Therefore sterilization of products by cathode rays can be achieved in a short period of time (a second or less). However, the penetration of cathode rays into matter (approximately 0.5 cm in material of unit density for each 2 mev) is much less than that of x-rays of corresponding voltage. The penetration of cathode rays into matter is expressed by the following equation:

$$R_{\max} = \frac{0.542E - 0.133}{\rho} \quad (\text{Eq. 1})$$

where  $R_{\max}$  is the maximum range (gm per cm<sup>2</sup>) for the cathode rays in matter of density  $\rho$ , and  $E$  is the voltage (megavolts) by which the cathode rays have been accelerated. Hence, unlike x- or  $\gamma$ -radiations, cathode rays have finite limits in matter.

*Neutrons.*—Neutrons are uncharged subatomic particles. They have relatively great penetration into matter, but they cannot be used for sterilization purposes because they can produce artificial radioactivity in material that would be treated by the neutrons. For example, when ordinary sulfur ( $S^{32}$ ) is bombarded with neutrons, it produces  $P^{32}$  which is radioactive and has a half-life of 14.3 days. When iron ( $Fe^{54}$ ) is bombarded with neutrons, it produces  $Fe^{55}$  which is radioactive and has a half-life of 4 years. Moreover, the production of neutrons is inefficient.

*Alpha Particles.*—Although alpha particles are densely ionizing radiations, they have little penetration into matter (like ultra-violet light) and hence cannot be considered for sterilization purposes.

Those radiations that may be considered for sterilization purposes, therefore, are limited to cathode rays (beta rays) or gamma radiations, the latter being predicated on the availability of large enough amounts of isotopes so that a sufficient quantity of radiation might be obtained to handle plant outputs.

## RADIATION STERILIZATION — BRIEF HISTORICAL ACCOUNT

Studies were made in the Department of Food Technology at the Massachusetts Institute of Technology by Proctor, Van de Graaff, and Fram (1943) which indicated that x-rays could destroy microorganisms in highly contaminated food material. With the end of the war and the subsequent availability of high-energy particle accelerators, the Department of Food Technology at MIT undertook an intensive program of investigation to study the potentiality and feasibility of applying ionizing radiations to the sterili-

zation of foods, drugs, and pharmaceuticals. The first aspects of this program of investigation involved a study of the fundamentals of ionizing radiations and fundamental studies of their effects on matter. As time went on, more laboratories have become interested in this field until, at the present time, there are at least ten separate groups throughout the country who are working in this field.\*

In the radiation studies conducted in these MIT laboratories from 1946 through 1950, a Van de Graaff particle accelerator (Trump and Cloud, 1943; Trump and Van de Graaff, 1948) was used as a source of ionizing radiations. In 1950, the United States Atomic Energy Commission announced the potential availability of immense quantities of energy in the form of radiations from radioactive isotopes, as by-products of atomic fission (Hayner and Proctor, 1953). A number of institutions were chosen to study the potentialities of utilizing isotopic sources of ionizing radiations for sterilization studies. Among these institutions was the Massachusetts Institute of Technology. Studies have been underway since 1950 in the Food Technology Laboratories at MIT to evaluate an isotopic source, namely, a kilocurie Cobalt-60 source (a "mock" fission source). More recently comparisons have been made in these laboratories on the relative efficiencies of radiations produced by machine sources and isotopic sources. These comparisons will be discussed later in this review.

## FUNDAMENTAL ASPECTS OF THE EFFECT OF IONIZING RADIATIONS ON MICROORGANISMS

### TARGET THEORY

In the main, ionizing radiations destroy microorganisms by direct hits of the radiations on or near the organism. This phenomenon has been reviewed in great detail by Lea (1947) and will be discussed here only briefly. It is believed that when a photon or a particle impinges on or comes close to a bacterial cell, it causes ionization in or near the bacterial cell, which results in the death of the organism.

The target theory may be illustrated by the following equation:

$$n/n_0 = e^{-D/D_0} \quad (\text{Eq. 2})$$

where  $n/n_0$  is the fraction of organisms surviving a dose  $D$ , and  $D_0$  is the mean lethal dose or 37 per cent dose, in other words, that

\*Most of the illustrations for this review are from the laboratory of the authors, chosen from the standpoint of both availability and convenience. Others may be found by reference to the literature cited.

dose of radiation that results in a survival of 37 per cent of the organisms ( $e^{-1}$ ).

The target theory states that when ionizing radiations bombard a culture of microorganisms, a first-order reaction results. The target theory may be recognized as the "*modus operandi*" if the following results are obtained:

(1) The survival curve is exponential.

(2) The concentration of organisms has no effect on the slope of the survival curve, *i.e.*, for a given dose, regardless of concentration, a certain percentage of microorganisms is destroyed for any given species.

(3) There is no temperature effect, *i.e.*, regardless of temperature, for a given dose of radiation of a particular species of microorganism the same percentage of the culture is destroyed.

(4) The rate at which the radiation is applied has no effect on the percentage of organisms affected.

#### INDIRECT ACTION THEORY

In general, direct hits on microorganisms are responsible for the death of the microorganisms. However, it has been shown by Proctor and Goldblith (1951*b*) and others that an indirect effect of radiations on the solute may have an effect on the microorganisms. In other words, some killing of bacteria and other microorganisms occurs as a result of free-radical production in the medium. Although this indirect action is less important than direct hits on the microorganisms, it has been shown to be of some significance.

### FUNDAMENTAL ACTION OF RADIATIONS ON SOLUTES OF BIOLOGICAL IMPORTANCE IN DILUTE SOLUTIONS

When one is considering the action of ionizing radiations on solutes in dilute solutions, one must realize that there are three important effects, the dilution effect, the protection effect, and the temperature effect. Each of these will be discussed separately, with illustrations chosen from the effects of ionizing radiations on biological materials of importance in food technology, such as nutrients, vitamins, and amino acids.

#### DILUTION EFFECT

When ionizing radiations bombard a solute in dilute solution, there is percentage-wise a greater destruction of the solute the more dilute the solution. Actually for a given dose of radiation the



same amount of solute is affected. However, percentage-wise the destruction of the solute is greater in the more dilute solution. Examples of this dilution effect with three different types of material are given in Table 122 and Figures 58 and 59. Table 122 illustrates the dilution effect with a vitamin, 1-ascorbic acid (Proctor and O'Meara, 1951). Figure 58 illustrates it with an

TABLE 122.—EFFECT OF 3 MEV CATHODE RAYS ON 1-ASCORBIC ACID AT VARIOUS CONCENTRATIONS\*

Sample	Total dose rep†	1-Ascorbic acid	
		mg/100 ml	Retention per cent
U.S.P. ascorbic acid in 0.25% oxalic acid			
A (control)	—	245	100
A-1	200,000	245	100
A-2	500,000	231	94.5
1 (control)	—	53.0	100
2	200,000	38.3	77.2
3	500,000	24.8	46.8
4 (control)	—	25.9	100
5	200,000	13.4	51.8
6	500,000	5.8	22.4
C (control)	—	4.8	100
C-1	200,000	0.64	13.3
C-2	500,000	Trace	—
Orange juice (con- centrated)			
3C (control)	—	196	100
4C	500,000	187	95.5
5C	1,000,000	177	90.3
6C	2,000,000	152	77.5
Orange juice (diluted 1:4)			
1D (control)	—	49.0	100
2D	500,000	31.4	64.0

\* Proctor and O'Meara, 1951.

† Roentgen-equivalents-physical.

enzyme, pepsin (Proctor, Goldblith, Bates, and Hammerle, 1952). Figure 59 illustrates it with an amino acid, dl-phenylalanine (Bhatia, 1950). In each of these three cases, for a given dose of radiation there was a greater effect, percentage-wise, in the more dilute solutions.

As the retention curves represent first-order reactions, the slopes of the curves can be calculated. The slope is known as the inactivation dose ( $D_0$  value.) For any given solute exposed to a specific type of radiation, the  $D_0$  value is less for the more dilute solutions. However, if one divides the  $D_0$  value for a compound

in solution of a given concentration by the concentration, a constant is obtained for that compound ( $D_0/C = K$ ). This is called the specific inactivation dose ( $D_0/C$ ) and is constant for a given solute exposed to a given type of radiation.

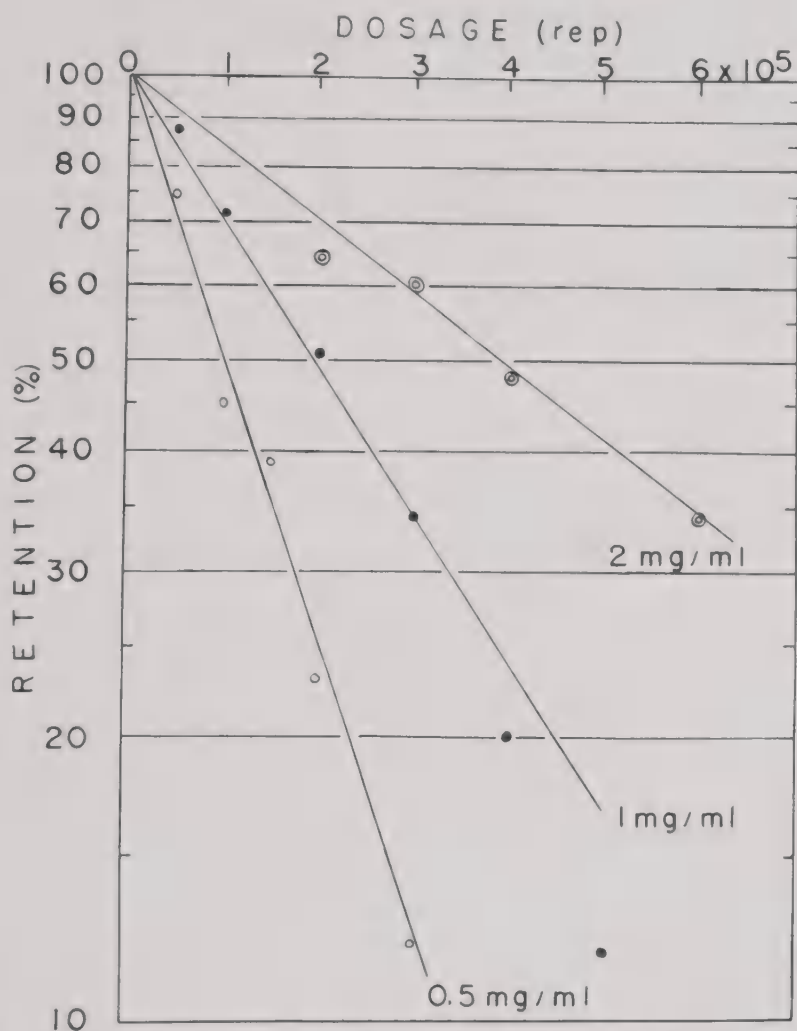


Fig. 58.—Effects of 3.0 mev cathode rays on aqueous solutions of pepsin of different concentrations. (Proctor, Goldblith, Bates, and Hammerle, 1952)

## PROTECTION EFFECT

Early in the studies in our laboratories on the effects of ionizing radiations on biological materials, it was found that although one might expect a certain quantum of destruction of a given solute in dilute solution as the result of a given dose of radiation, if another solute were added to the solution the effect might become

TABLE 123.—EFFECT OF HIGH-VOLTAGE X-RAYS ON SOLUTIONS OF U. S. P. NIACIN, U. S. P. ASCORBIC ACID AND ON MIXTURES OF THE TWO\*

Sample	Concentration of		Time of irradiation sec	Dose		Concentration after irradiation		Retention after irradiation	
	Niacin $\gamma$ /ml	Ascorbic acid $\gamma$ /ml		Total roentgens	Rate r/sec	Niacin $\gamma$ /ml	Ascorbic acid $\gamma$ /ml	Niacin per cent	Ascorbic acid per cent
4	50	500	—	Control	—	50.0†	490.0†	—	—
6	50	500	205	125,000	599	22.0	419.0	44.0	83.8
16	50	—	287	125,000	436	43.0	—	86.0	—
27	—	500	270	125,000	463	—	333.0	—	66.6
15	50	500	542	250,000	462	12.5	392	25.0	78.4
17	50	—	542	250,000	462	20.0	—	40.0	—
1	—	500	500	250,000	453	—	279	—	55.8

\* Proctor and Goldblith, 1948. † Not irradiated.

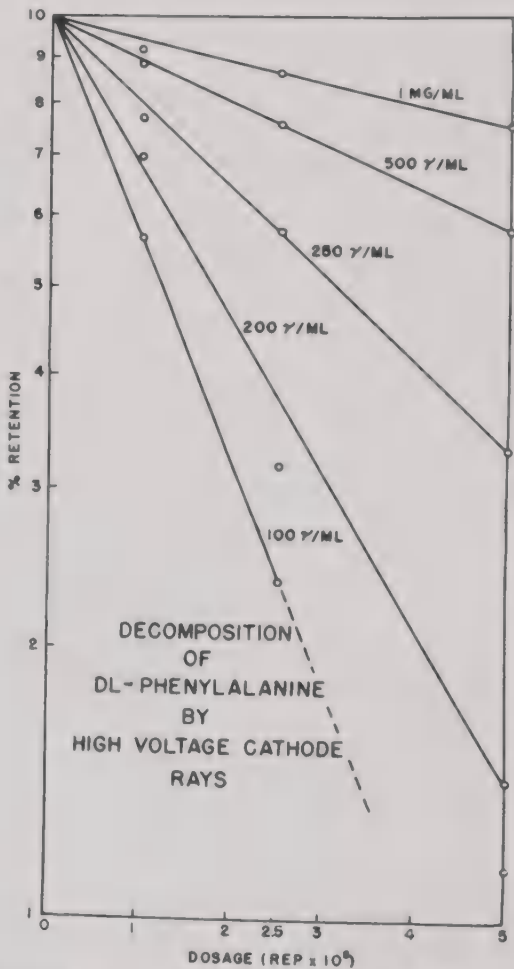


Fig. 59.—Effects of cathode rays on aqueous solutions of dl-phenylalanine of different concentrations. (Bhatia, 1950)



entirely different. For example, Table 123 (Proctor and Goldblith, 1948) indicates that, whereas niacin is a relatively stable compound and ascorbic acid a rather radiosensitive compound when each is irradiated alone, the niacin protects the ascorbic acid when the two are mixed and irradiated together. This phenomenon is known as the protection effect. This effect is discussed in some detail by investigators in the field, such as Dale, Davies, and Meredith (1949), Forssberg (1950), and Proctor, Goldblith, Bates, and Hammerle (1952).

The protection effect may be described as follows: Whereas a given quantity of solute is affected by a given dose of radiation, if another substance is added to that solute, the added substance

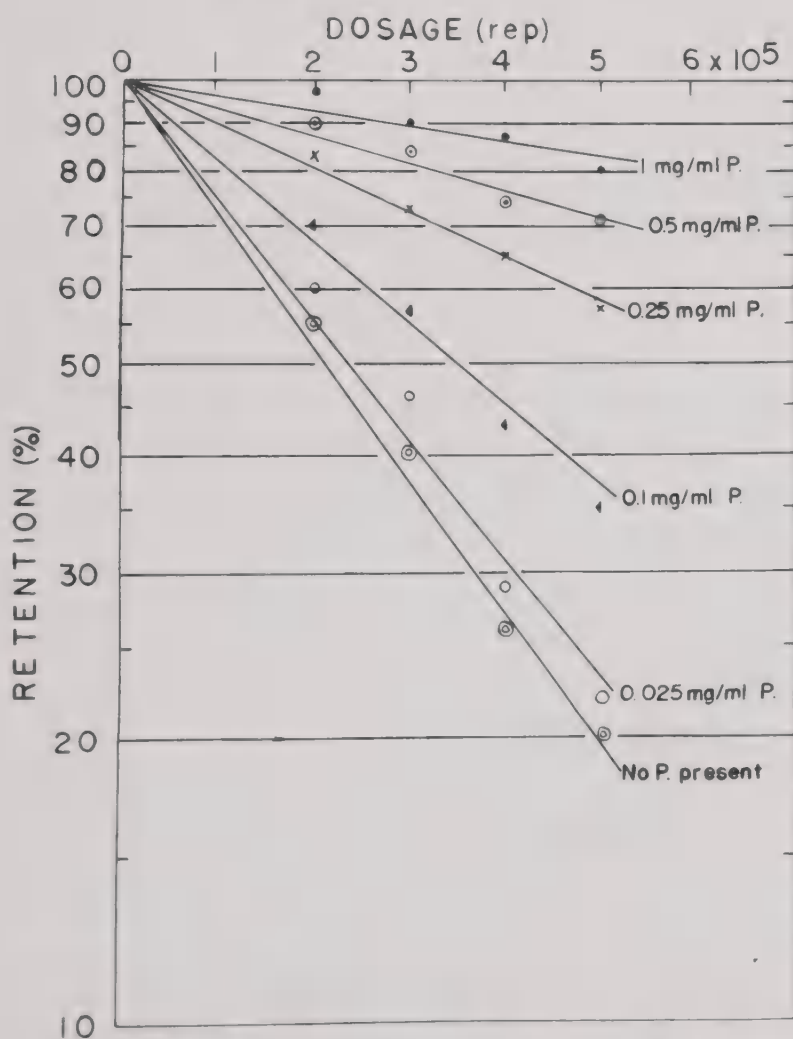


Fig. 60.—Effects of 3 mev cathode rays on crystalline pepsin in acetate buffer (pH 4.3) and in the presence of varying quantities of sodium d-isoascorbate. (Proctor and Goldblith, 1952b)

may itself become affected by the radiation and protect the original indicator-solute in question.

Illustrations of the protection effect, based on observations made in our laboratories (Proctor and Goldblith, 1952*b*) are given in Figures 60 and 61. Figure 60 illustrates the effect of high energy cathode rays on pepsin with sodium-d-isoascorbate as a protective

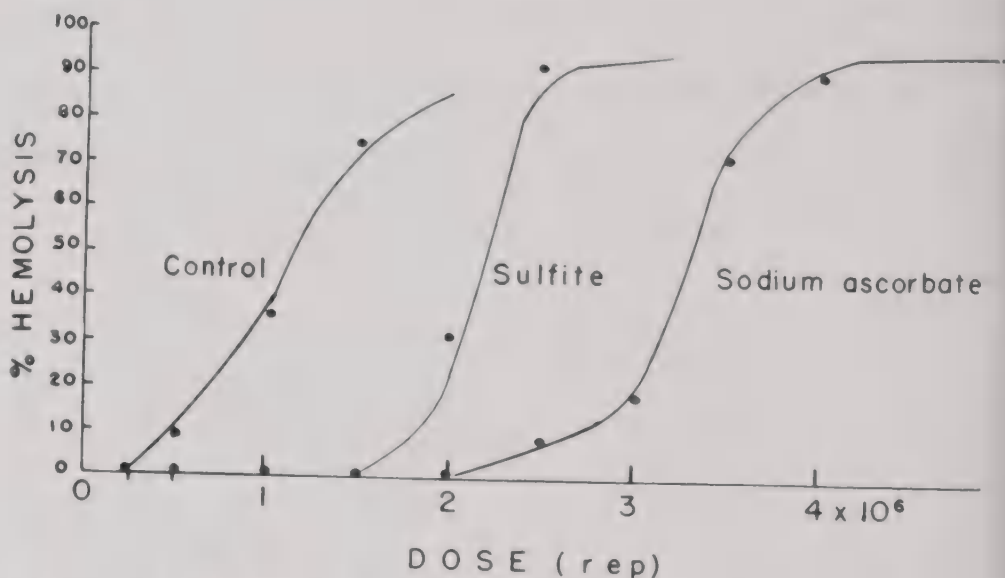


Fig. 61.—Effect of cathode rays on hemolysis of 5 per cent red blood cells (bovine) in physiological saline. (Proctor and Goldblith, 1952*b*)

substance. Figure 61 shows the effectiveness of sodium-d-isoascorbate as a protective compound for irradiated red blood cells.

The protection may be expressed mathematically (Dale, Davies, and Meredith, 1949) as follows:

$$Q = \frac{D_{O(I+P)} - D_{O(I)}}{D_{O(I)}} \times \frac{C_I}{C_P} \quad (\text{Eq. 3})$$

In Equation 3,  $Q$  is the protective quotient,  $D_{O(I+P)}$  is the inactivation dose (37 per cent dose) for the indicator plus protector,  $D_{O(I)}$  is the inactivation dose for the indicator alone,  $C_I$  is the concentration of the indicator that is present, and  $C_P$  is the concentration of the protector that is present. In Figure 60, the indicator was pepsin and the protective compound, sodium-d-isoascorbate.

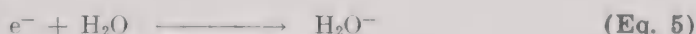
Compounds that have a  $Q$  value greater than 1 are of interest in protecting certain substances with which one may be concerned,

and those that have a  $Q$  value of 5 or greater are of considerable interest. A  $Q$  value of 5 means that the particular protective compound has an affinity five times as great as the indicator compound for the radiation.

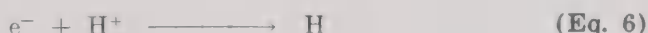
It would be well at this time to consider exactly how ionizing radiations act when bombarding dilute solutions of solutes. Ionizing radiations lose energy, in part at least, when they pass through water, by causing water molecules to ionize:



The ejected electron gets away from the positive water ion and becomes attached eventually to another molecule, when a negative water ion is formed:



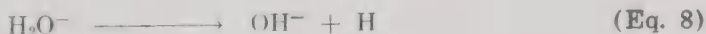
On the other hand, the ejected electron may combine (although probably it rarely does so) with a hydrogen ion and form a free hydrogen atom:



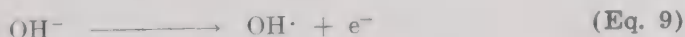
The positive water ion interacts with its neighboring water molecules and thereby leads to dissociation:



The negative water ion (Equation 5) dissociates:



In addition to the reaction illustrated in Equation 7, ejection of electrons from hydroxyl ions normally present in water may result in the formation of a hydroxyl radical.



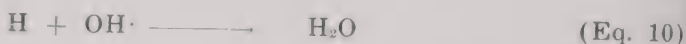
The electrons produced in this manner may yield hydrogen atoms, according to Equation 6.

In the reaction of ionizing radiations with water, the important products formed are the  $\text{OH}\cdot$  and the  $\text{H}$  atom.



The  $\text{OH}\cdot$  is a strong oxidizing agent, and the H atom is a powerful reducing agent. Hence any oxidizable solutes present will be oxidized by these radiations through the  $\text{OH}\cdot$  produced, and any reducible solutes will be reduced through the H atoms produced.

If the H atom and the  $\text{OH}\cdot$  are close enough together (Equation 10), they may recombine, in which case there will be no net change:



On a molecular scale, however, the distance between the hydrogen atom and the hydroxyl radical is so great that before

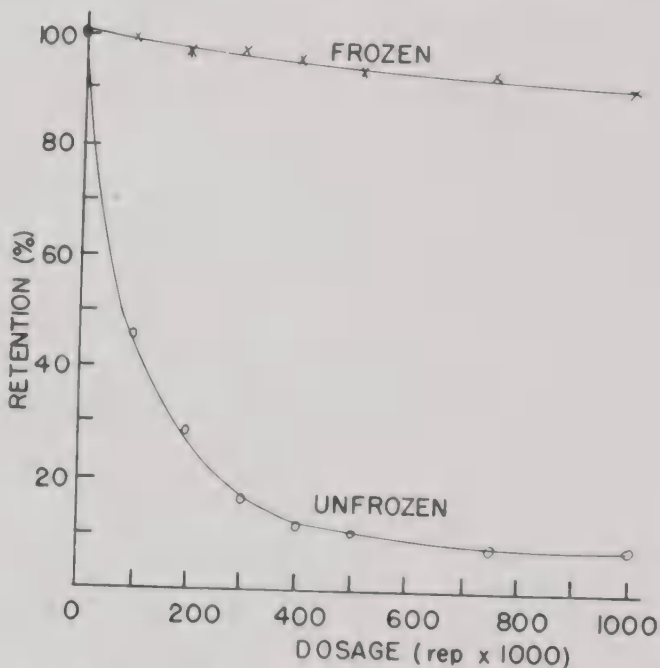


Fig. 62.—Effects of 3 mev cathode rays on frozen and non-frozen solutions of ascorbic acid in 0.25 per cent oxalic acid. (O'Meara, 1949)

diffusion brings them together something else may happen, such as the oxidation of an oxidizable solute or the reduction of a reducible solute.

The fundamental effect of ionizing radiations on solutes in dilute solution, therefore, is to produce free radicals having either an oxidation or a reduction reaction. These free radicals are of great practical importance as the causative agents of off-flavor production in foods being bombarded by ionizing radiations. This subject will be treated in more detail subsequently.

## TEMPERATURE EFFECT

The indirect action theory postulates that when solutes are in a frozen state ionizing radiations have little or no effect on them, because the free radicals produced by the radiations are unable to diffuse into and affect the solute molecules. This phenomenon is illustrated by the curves in Figure 62, showing the effect of 3 mev cathode rays on frozen and non-frozen solutions of ascorbic acid in 0.25 per cent oxalic acid, and the curves in Figure 63, showing the effect on ascorbic acid in frozen and non-frozen fresh orange juice (O'Meara, 1949). It is to be observed that, although the

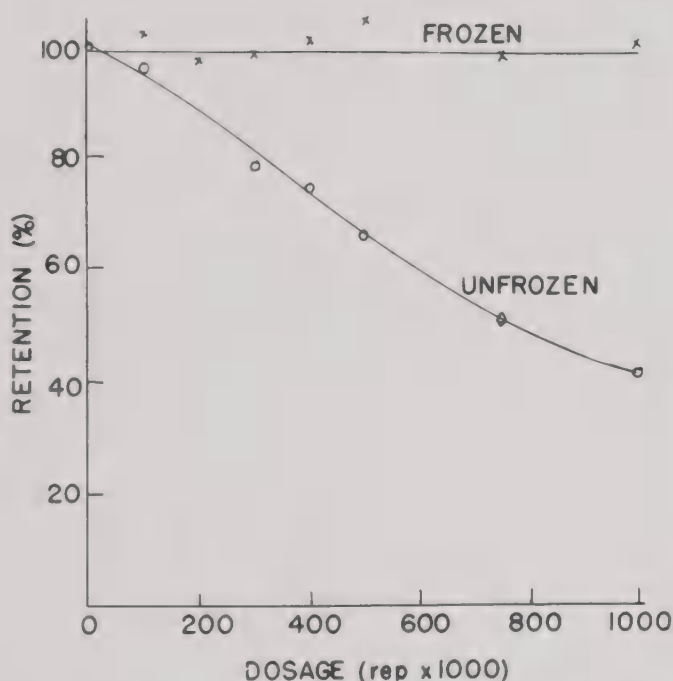


Fig. 63.—Effects of 3 mev cathode rays on ascorbic acid in frozen and non-frozen fresh orange juice. (O'Meara, 1949)

ascorbic acid in the liquid solutions was readily destroyed by relatively low doses of radiations, the ascorbic acid in the frozen solutions was not affected.

## APPLICATION OF FUNDAMENTALS OF IONIZING RADIATIONS TO PROCESSING OF FOODS AND DRUGS

### THE ORGANISM

Of vital importance in any consideration of the processing of materials by ionizing radiations are the effects of these radiations on the microorganisms responsible for spoilage of the

materials. The various factors that play roles in the radiation effects will be discussed separately.

*Concentration of Microorganisms.*—As explained previously, the concentration of a given species of microorganism in any material does not affect the percentage of microorganisms of this species destroyed by a given dose of radiation. However, to reduce different concentrations of microorganisms of a given species in different materials (or in several samples of the same material) to the same

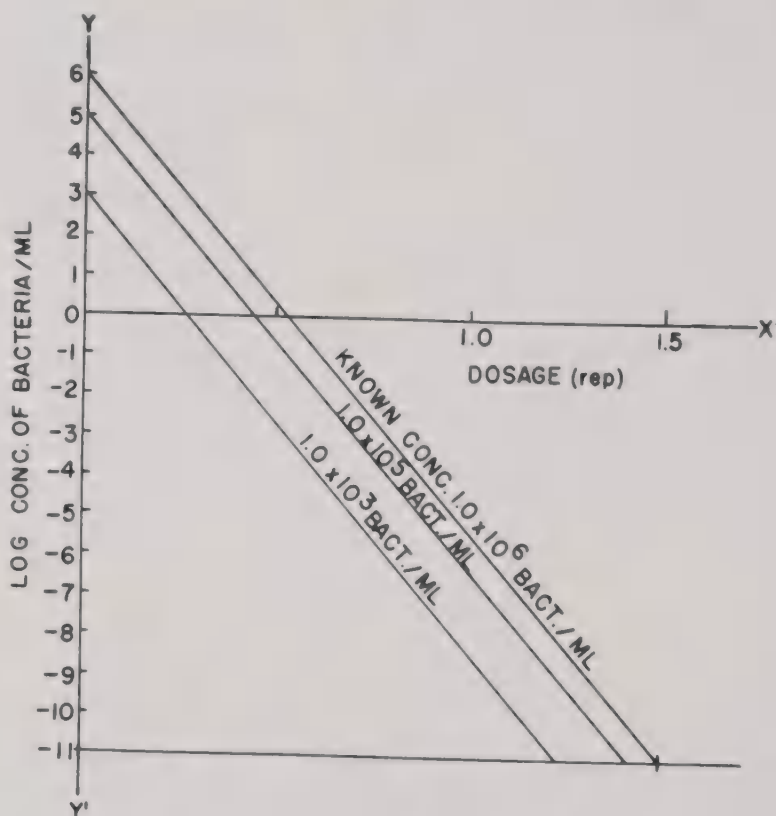


Fig. 64.—Curves illustrating differences in doses of cathode rays required to reduce different concentrations of microorganisms of a given species to the same level of sterility. (Proctor and Goldblith, 1952a)

low level after irradiation, a greater dose of radiations is required for those materials having the greater initial concentrations of the microorganism. This is illustrated in Figure 64. Nevertheless, although the concentration of microorganisms plays a role in the dose required to effect a given degree of sterilization, this role is not an appreciable one. For example, Sample A having a concentration of microorganisms a thousandfold greater (that is,  $\log = 3$ ) than that of Sample B requires only a slightly larger dose percentage-wise for sterilization than that required for Sample B.



The reason for this is that the reaction is a first-order reaction (log of concentration vs. arithmetic dose).

*Radiosensitivity of a Given Species.*—The individual radiosensitivity of a given species of microorganisms is by far the most important single factor in the radiation effect. It has been well established by investigators in our laboratories (Goldblith *et al.*, 1953*b*) and elsewhere that to effect a given degree of sterility a much greater dose of ionizing radiations is required for spore-forming organisms than for vegetative organisms, and that the vegetative organisms vary among themselves in their radiosensitivities. For example, a spore-former such as *B. thermoacidurans*

TABLE 124.—RELATIVE RADIOSENSITIVITIES OF 11 SPECIES OF BACTERIA EXPOSED TO GAMMA RAYS OF KILOCURIE COBALT-60\*

( $10^8$  organisms per ml initial concentration)

Organism	$tD_0$ values
<i>Escherichia coli</i>	6.5 mins
<i>Aerobacter aerogenes</i>	4.8 "
<i>M. pyogenes</i> var. <i>aureus</i>	20 "
<i>M. pyogenes</i> var. <i>albus</i>	13 "
<i>Streptococcus faecalis</i>	28 "
<i>Serratia marcescens</i>	4.3 "
<i>Corynebacterium xerose</i>	18 "
<i>Ps. fluorescens</i>	2.8 "
<i>Ps. aeruginosa</i>	2.7 "
<i>Sarcina lutea</i>	15 "
<i>Bacillus thermoacidurans</i>	39 "

\* Dose rate, 1640  $r_{93}$  per minute  
(Goldblith *et al.*, 1953*b*)

is much more radioresistant than a vegetative organism such as *E. coli* (Table 124).

The effect of this difference in radiosensitivity on the sterility dose requirement is illustrated in Figure 65. Here it can be seen that a five-fold greater resistance of a bacterial species necessitates a five-fold greater dose of radiations to effect the same degree of sterility.

Organisms are classified as to their radiosensitivity or conversely their radioresistance according to the so-called mean lethal dose ( $D_0$ ) required under given conditions to effect the destruction of 63 per cent (a survival of 37 per cent,  $e^{-1}$ ) of the original concentration of organisms. An organism for which the  $D_0$  value is 50,000 r would require a sterility dose ten times that of an organism for which the  $D_0$  value is 5,000 r (*e.g.*, *B. thermoacidurans* vs. *E. coli*).

*Temperature of Irradiated Material.*—It has been assumed for a long time that the temperature of the material being irradiated should have little effect on the number of microorganisms destroyed. Although this has been found to be true in the majority of cases, a small amount of data (Table 125) suggest that such might possibly not be entirely correct. This point is certainly worthy of further investigation.

TABLE 125.—EFFECT OF FREEZING AND IRRADIATION BY SUPERVOLTAGE CATHODE RAYS ON THE SURVIVAL OF MICROORGANISMS IN RAW MILK\*

Dose rep	Non-frozen milk		Frozen milk	
	Bacteria per ml	Survival per cent	Bacteria per ml	Survival per cent
Control	275,000	—	300,000	—
100,000	9,000	3.3	30,000	10.0
200,000	700	0.25	9,000	3.0
300,000	130	0.05	2,700	0.9
500,000	5	0.00	34	0.01
750,000	0	0	4	0
1,000,000	0	0	0	0

\* Proctor and Goldblith, 1951a

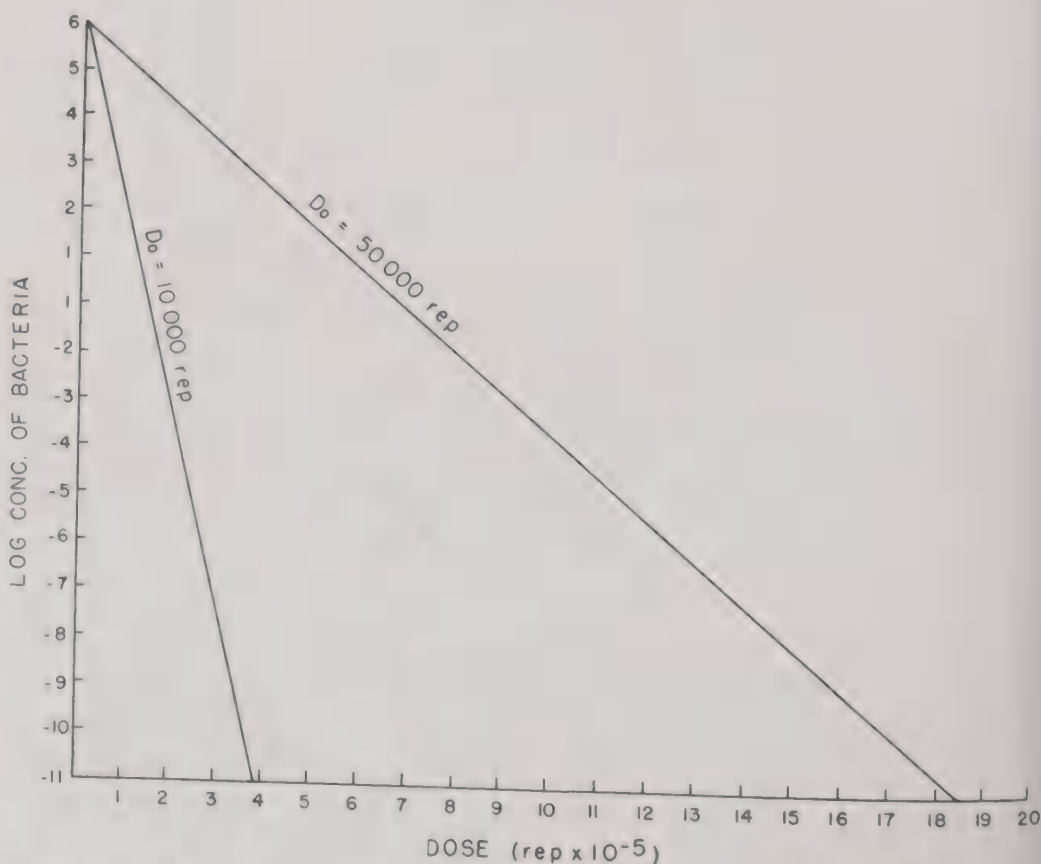


Fig. 65.—Effect of the resistance of a bacterial species as represented by the mean lethal dose ( $D_0$ ) on the sterility dose requirement.

*Vehicle or Menstruum.*—That the indirect effect of radiations on the solute may have an influence on microorganisms in the solute has been discussed previously. It is sufficient to state at this point that in any irradiation studies made on any given spoilage organism the particular medium to be treated must be inoculated with the spoilage organism in adequate concentration. This medium may have either an inhibitory effect on the microorganisms or may be conducive to their growth and may protect the microorganisms from the radiations. Therefore, it is necessary that an inoculation study be made with the particular vehicle that is to be treated. An illustration of the effect of the medium is given in Table 126.

TABLE 126.—DEPENDENCE OF SURVIVAL OF *S. aureus* 209 ON PROTEIN CONCENTRATION OF THE VEHICLE (FRACTIONATED DOSAGE OF CATHODE RAYS)\*

Protein concentration of vehicle gm/ml	Fraction of microorganisms surviving cathode ray dose (in rep)			
	$5 \times 10^3$	$10 \times 10^3$	$15 \times 10^3$	$20 \times 10^3$
0	0.78	0.37	0.23	0.12
$10^{-7}$	0.74	0.47	0.31	0.10
$10^{-6}$	0.68	0.44	0.29	0.16
$10^{-5}$	0.73	0.49	0.31	0.15
$10^{-4}$	0.78	0.57	0.45	0.22
$10^{-3}$	0.79	0.61	0.48	0.25
Nutrient broth	0.80	0.65	0.52	0.42

\* Proctor and Goldblith, 1951b

*Oxygen Tension.*—Hollaender *et al.* (1951) and others have shown that the rate of destruction of microorganisms by ionizing radiations is affected, in part at least, by the oxygen tension that is available. Hollaender and his colleagues found that *E. coli* was more resistant to a nitrogenous atmosphere and in a vacuum than in an air atmosphere. This is of great importance in the practical consideration of processing by ionizing radiations.

*Summary.*—This consideration of the factors that play roles in the effects of ionizing radiations on microorganisms emphasizes an important point insofar as concerns the application aspects of these radiations. It is of extreme importance to conduct fundamental studies on the effects of radiations on microorganisms under the same conditions (regarding environment and other factors) as those that would prevail during actual processing.

#### EFFECTS OF RADIATIONS ON PRODUCTS

*Nutrients.*—It has been shown that ionizing radiations have an effect on any single nutrient in dilute solutions. However, most materials that would be considered for processing by this means are complex materials, containing large numbers of solutes. Such



materials as foods or pharmaceuticals, for example, are composed of several complex molecules, and it has been found that ionizing radiations have little effect on any particular solute (Proctor and Bhatia, 1950) because of the dilution and protection phenomena. It will be observed from Table 127 that ionizing radiations had no

TABLE 127.—EFFECT OF CATHODE RAYS ON AMINO-ACIDS IN HADDOCK FILLETS\*

Amino acid and dose of cathode rays	Amino acid content†		Change in amino acid (per cent)	
	Control	Irrad.	Loss	Gain
<i>Phenylalanine</i>				
900,000 rep	3.63	3.73	0.00	2.75
2,700,000 rep	3.86	3.77	2.33	0.00
5,700,000 rep	3.93	3.69	6.10	0.00
<i>Tryptophan</i>				
900,000 rep	1.06	1.02	3.77	0.00
2,700,000 rep	1.02	0.92	9.80	0.00
5,700,000 rep	1.16	1.08	6.92	0.00
<i>Methionine</i>				
900,000 rep	2.86	2.84	0.70	0.00
2,700,000 rep	2.85	2.69	5.61	0.00
5,700,000 rep	2.99	2.85	4.68	0.00
<i>Cystine</i>				
900,000 rep	1.02	0.99	2.94	0.00
2,700,000 rep	1.01	0.94	6.93	0.00
5,700,000 rep	1.04	1.04	0.00	0.00
<i>Valine</i>				
900,000 rep	6.42	6.55	0.00	2.03
2,700,000 rep	6.32	6.24	1.27	0.00
5,700,000 rep	6.29	6.69	0.00	6.36
<i>Leucine</i>				
900,000 rep	7.89	7.74	1.90	0.00
2,700,000 rep	7.92	7.44	6.06	0.00
5,700,000 rep	8.03	8.25	0.00	2.74
<i>Histidine</i>				
900,000 rep	1.98	1.97	0.50	0.00
2,700,000 rep	2.02	1.85	8.41	0.00
5,700,000 rep	1.85	2.00	0.00	8.11
<i>Arginine</i>				
900,000 rep	5.61	5.48	2.32	0.00
2,700,000 rep	5.63	5.53	1.78	0.00
5,700,000 rep	5.34	5.56	0.00	4.12
<i>Lysine</i>				
900,000 rep	10.47	9.80	6.40	0.00
2,700,000 rep	9.52	9.52	0.00	0.00
5,700,000 rep	9.70	9.29	4.23	0.00
<i>Threonine</i>				
900,000 rep	4.37	4.41	0.00	0.91
2,700,000 rep	4.13	4.29	0.00	3.87
5,700,000 rep	4.87	4.58	5.95	0.00

\* Proctor and Bhatia, 1950.

† Amino acid content expressed as parts of amino acid per 16 parts of nitrogen

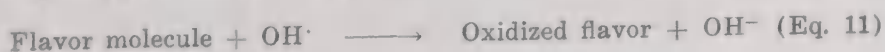
significant effect on the particular essential amino acids studied in irradiated haddock fillets, whereas it has been found that similar doses of radiation have measurable and pronounced effects on the essential amino acid content in dilute solutions.

Although it has been shown that ionizing radiations have little effect on nutrients, further research is necessary to prove conclusively that the wholesomeness of foods, that is, the biological value of the foods to animals, is not changed by irradiation. In other words, research is necessary to demonstrate that an irradiated food is equally as good as a non-irradiated food for supporting the growth of animals over long periods. Furthermore, the term "wholesomeness" means that a toxic compound is not produced by the radiations. Although there is no reason to suspect at the present time that ionizing radiations may produce a toxic compound in irradiated foods, it is believed that animal tests should be conducted to make certain that food materials or drugs are safe for consumption after radiation bombardment.

*Side Reactions.*—It has been shown by all investigators in the field that when food materials or other absorbers are treated with ionizing radiations, undesirable side effects resulting from the formation of free radicals are produced. Among the undesirable side effects are changes in color and the production of off-flavors and off-odors. Because such side effects are highly detrimental to the product, any type of processing that affects a product in such ways would not be used. It is highly important, therefore, that the problems regarding these undesirable side reactions be overcome.

A program of investigation has, therefore, been initiated in the Department of Food Technology at the Massachusetts Institute of Technology with the object of attempting to prevent undesirable side effects by the application of fundamental information obtained in earlier research. According to this program of research, which has been discussed in detail by Proctor and Goldblith (1952*b*) and by Proctor, Goldblith, Bates, and Hammerle (1952), the effect of addition of a free-radical acceptor to the material to be irradiated is being studied. A free-radical acceptor is a compound that protects against side reactions by having a greater affinity for the free radicals produced by ionizing radiations than is possessed by the flavor or color molecules of the irradiated material (a *Q* value greater than unity).

In irradiated food materials, off-flavors are produced that are characterized by some members of taste panels as "oxidized." What conceivably happens may be explained schematically in Equation 11:







are little affected, certain types of films made of polymeric substances might conceivably be affected by ionizing radiations. Therefore, further investigation in this field is necessary.

# ABSORPTION OF RADIATIONS IN DIFFERENT ABSORBERS

*Cathode Rays.*—Cathode rays produce a non-uniform ionization in matter (Trump, Wright, and Clarke, 1950). When cathode rays bombard matter, there is a greater intensity of ionization at one-third of the maximum range of the particular energy of cathode rays beneath the surface than at the surface of the absorber (Fig. 66). This is due to the inherent scattering of cathode rays

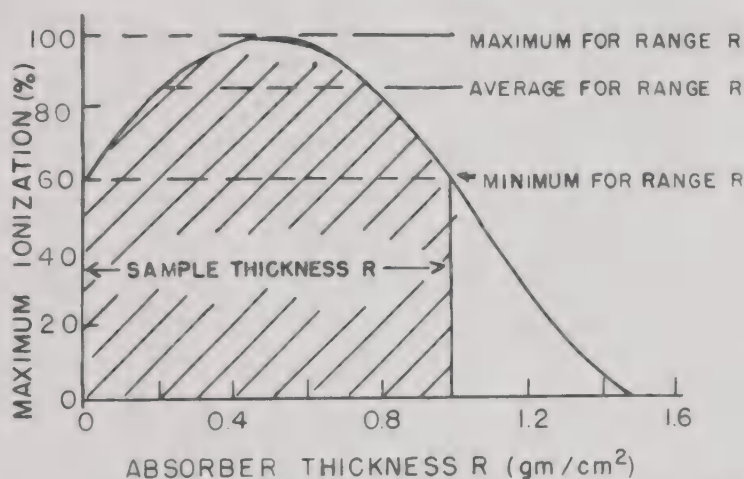


Fig. 66.—Ionization in depth of cathode rays for maximum, minimum, and average doses. (Trump, Wright, and Clarke, 1950)

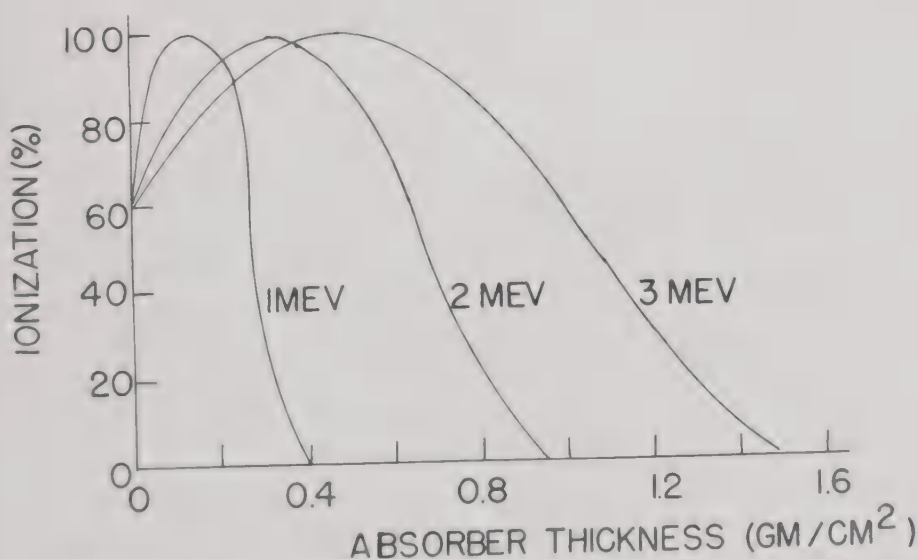


Fig. 67.—Distribution of ionization in depth of aluminum produced by cathode rays of different energies. (Proctor and Goldblith, 1952a)

when they bombard matter. The effect of increasing the voltage of cathode rays is illustrated in Figure 67 (Proctor and Goldblith, 1952a). Discussion of the efficiency of utilization of such beams is beyond the scope of this present treatment, important though the subject is. For further information, the reader is referred to a detailed study reported by Goldblith and Proctor (1952).

*Gamma or x-Rays.*—Gamma or x-rays have an ionization-in-depth curve (Fig. 68) entirely different from that of cathode rays. Whereas cathode rays have a finite penetration into matter, gamma

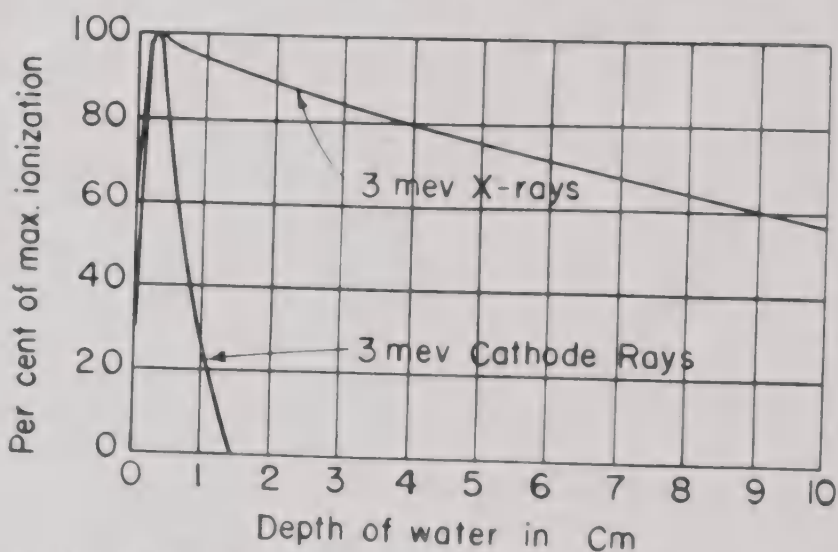


Fig. 68.—Distribution of ionization in water by 3 mev x-rays and cathode rays (Trump and Van de Graaff, 1948)

or x-rays have an exponential penetration expressed by the typical Beer-Lambert Law:

$$I = I_0 e^{-\mu x} \quad (\text{Eq. 15})$$

where  $I_0$  is the quantity of radiation impinging on a given surface of thickness  $x$ ,  $I$  is the quantity of radiation emitting after passing through thickness  $x$ , and  $\mu$  is the linear absorption coefficient for the particular quality of radiation. Theoretically, with a beam of x- or gamma radiation, the dose never diminishes to zero. For practical considerations, however, it is obvious that this is not the case. The importance to food processing of the heterogeneous ionization of cathode rays as compared with the homogeneous ionization of x- or gamma rays is the subject of a paper by the authors and other researchers in these laboratories (Goldblith *et al.*, 1953a). With a homogeneous beam of ionization such as one gets with x- or gamma rays, a given dose of radiation will result

in a greater percentage of microorganisms killed than is the case with a non-uniform ionization beam such as one obtains with cathode rays. For further information on this subject, the reader is referred to the paper cited above.

### SOURCES OF RADIATIONS

*Machine.*—Particle accelerators producing up to 12 kilowatts of energy are available today. A realization of the quantity of energy involved in 12 kilowatts may be had by consideration of the following relationships:

$$\begin{aligned} 10^6 \text{ rep} &= 8.3 \text{ joules per gram} \\ 1 \text{ joule} &= 1 \text{ watt-second} \\ 1 \text{ rep} &= 83 \text{ ergs per gram} \end{aligned}$$

TABLE 128.—GENERAL FACTORS OF IMPORTANCE IN ELECTRON ACCELERATORS USED FOR STERILIZATION PURPOSES

Voltage (mev)	Determines penetration
Electron beam current (ma)	Determines number of electrons per unit of time bombarding sample, hence rate of sterilization of material of thickness determined by voltage
Total power (watts)	Combination of voltage and current that determines total quantity of material that can be sterilized
Efficiency of utilization (per cent)	Based on inherent variation of ionization in depth of cathode rays in matter
Reliability of operation	Determines how much material needs to be re-processed; at present appears to be limited, in general, by tube performance; dictates number of spare machines required
Cost of sterilization (cents per lb)	Determined by original capital investment, over-all performance sterilization units, number of units required, cost and frequency of replacement of certain components

From these relationships, the roentgen or roentgen-equivalent-physical unit (rep) can be converted into joules and watts, the conventional fundamental units of power.

A number of factors must be taken into consideration when machines or particle accelerators are to be used as sources of ionizing radiation for sterilization purposes. Among these factors are voltage, current, power, efficiency of utilization, reliability of operation, and cost of sterilization (Table 128). Just as with any other type of processing, uniformity of dose must be achieved with the machine used to produce ionizing radiations. This means that the output of an accelerator must remain as constant as possible, with little variation in voltage or current for a given period of time. If there is any appreciable decrease in either of these power



components, the material being processed at that time would have to be reprocessed.

In general, a great deal of energy is available from ionizing radiations in the form of cathode rays. The production of x-rays by particle accelerators is inefficient. Therefore, when one is considering machine sources of radiation, one is speaking of producing cathode rays.

At the voltage levels available today, the penetration of cathode rays into matter is much less than that required for conventional food containers. It has been shown (Proctor and Goldblith, 1951b) that a can of corned beef can be sterilized by 6 mev cathode rays by a technique known as "cross-firing" or firing from two directions. However, 6 mev cathode rays, although achievable in the laboratory today, have not been produced with the necessary characteristics for commercial accelerators. Further research along this line is necessary.

*Isotopic Sources.*—With gamma-emitting isotopes it is possible to achieve the necessary penetration into large containers of conventional type, such as sanitary cans and large vials. However, it is also necessary to achieve a dose rate sufficient for rapid sterilization on a commercial scale. From this standpoint it is estimated that for a penicillin-producing plant of medium size, for instance, approximately 50 mega-curies of radioactive isotope would be necessary to process the output of the plant. This represents a great quantity of isotopes. Moreover, it should be remembered that an isotopic source of radiation can never be shut off, and hence great care must be taken to protect the personnel. Obviously any exposures of material must be done by remote control, and this may be a costly procedure.

Other factors of importance in the consideration of gamma-emitting isotopes mentioned previously (such as the uniformity of ionization produced) make these isotopes desirable, however. At the time this review article was written, the Atomic Energy Commission had not stated when it expects sufficient isotopes will be available for sterilization purposes or what quantity of these isotopes will be available. For the present, therefore, one can merely conduct research, hoping that sufficient quantities of isotopes in the mega-curie range will become available for sterilization purposes.

#### PROTECTION OF PERSONNEL

One of the requirements in the application of ionizing radiations to processing is the protection of personnel. The health-physics aspects in the use of such radiations will not be discussed in detail

here. However, it should be pointed out that an employer who installs either a machine or an isotopic source of radiation in a plant must provide safeguards for personnel who may be working directly with the equipment or who may be working in a place adjacent to the particular source of radiation. Such protections as periodic physical examinations, blood counts, the carrying of ionization chambers and film badges, and proper shielding for the particular accelerator must be considered. These types of protection have been discussed in some detail elsewhere (Proctor and Goldblith, 1951*b*, 1952*a*).

## COSTS

The costs of radiation sterilization have been discussed previously (Proctor and Goldblith, 1951*b*; Stanford Research Institute, 1951). Costs such as those represented by the initial investment, replacement parts, electricity, and personnel are the same as those encountered in conventional processing. In addition, there are costs peculiar to this particular system of sterilization, such as health-physics costs, protection and shielding costs, and structural changes of the building to allow for increased shielding.

The actual cost of radiation sterilization per pound of material can be calculated by totalling the costs of the various components (accelerators, building changes, and cost of running the equipment per pound of material) and dividing by the total number of pounds of material sterilized over a given amortization period. It is felt by those concerned with the manufacture of equipment that this type of sterilization should cost only a small fraction (one-tenth of a cent or less) per pound of material. However, further study of the reliability of particle accelerators is necessary before actual costs can be figured more closely.

## RESEARCH DEVELOPMENTS NECESSARY TO MAKE COLD STERILIZATION A REALITY

### ON THE SOURCE OF RADIATION

Although particle accelerators are fabricated by several manufacturers, no particular piece of equipment has as yet been produced the reliability of which is known under commercial operating conditions. Therefore, one of the greatest requirements at the present time, so far as particle accelerators are concerned, is to obtain satisfactory information on how long an accelerator can operate without breaking down, without a drop in voltage, and without a loss in current.

The second requirement, in the case of machine sources of ionizing radiations (cathode rays), is to obtain energy levels higher than 3 mev. Many small pharmaceutical ampuls can be sterilized by radiations produced by machines now available. The surface sterilization of a number of products can also be achieved. However, energy levels of the order of 5 to 10 mev would be necessary for the conventional type of containers now being used in the food and drug industries.

To summarize, it is important so far as machine sources of radiation are concerned to study the reliability of presently available equipment, to improve that reliability if necessary, and to achieve greater energy outputs of ionizing radiations as reflected by increases in the accelerating voltage of the particles.

### ON THE PRODUCTS

A great deal of study is still necessary to prevent undesirable side effects on all products under consideration for sterilization. Research is also necessary to study, under proper supervision, the wholesomeness of irradiated foods with respect to any possible toxic effect. Further research is likewise necessary with packaged foods inoculated with spoilage organisms and with a large number of pharmaceuticals that might be sterilized by ionizing radiations.

### FOOD AND DRUG MATERIALS OFFERING POTENTIALITIES FOR STERILIZATION BY IONIZING RADIATIONS

Among food materials and drugs offering potentialities for sterilization by ionizing radiations are those that have a high selling price (hence a greater differential between cost and selling price) which would cover the increased cost that occurs at present in sterilization by this means. On this basis it would appear more practical to sterilize meats by ionizing radiations than vegetables, which have a much lower selling price and cost-price differential. In view of the success that appears to be obtainable in the irradiation of meats, these products would seem to be among the most promising foods for sterilization by ionizing radiations. Other products of interest are grains. Prevention of reproduction of the lesser grain borer, the rice weevil, and other insects which damage millions of dollars' worth of grain each year can be prevented by low doses of ionizing radiations (100,000 rep or less). Therefore, one of the more promising fields of use of cold sterilization may be that of protection of grains against insects.

Among the drugs, insulin has been found to be adversely affected by ionizing radiations, but a number of heat-sensitive



pharmaceuticals such as penicillin (Table 129) and streptomycin (Huber, 1948) have been shown to be efficaciously sterilized by this means. Therefore, it would appear that in the drug field the utilization of ionizing radiations for sterilization has potentialities.

Successful sterilization of segments of human aortae for subsequent transplant into patients suffering from coarctation of the aorta has been achieved recently (Meeker and Gross, 1951), based on fundamental studies made in our laboratories (Proctor and Goldblith, 1951a). Today more than twenty successful transplants have been made in humans in the Boston area. These studies indicate great potentialities for sterilization of other types of human tissues by use of ionizing radiations.

TABLE 129.—BIOLOGICAL EFFECT OF SUPERVOLTAGE CATHODE RAYS  
ON PHARMACEUTICALS

Product	Dose rep	Bacterial spore contamination		Loss in potency after irradiation
		Before irradiation	After irradiation	
<i>K. penicillin</i>	$4.1 \times 10^6$	yes	no	no loss
Ergotrate	$4.1 \times 10^6$	yes	no	50 per cent
Ergotrate	$0.5 \times 10^6$	—	—	no loss
Thromboplastin	$4.1 \times 10^6$	yes	no	100 per cent
Insulin	$4.1 \times 10^6$	yes	no	98 per cent
Insulin	$0.1 \times 10^6$	—	—	100 per cent
Protamine zinc insulin	$4.1 \times 10^6$	yes	no	100 per cent
Protamine zinc insulin	$0.1 \times 10^6$	—	—	75 per cent
Thiobarbiturate	$4.1 \times 10^6$	yes	no	slight loss
B complex + Vit. C	$4.1 \times 10^6$	yes	no	no loss
Duracillin in oil	$4.1 \times 10^6$	yes	no	no loss
Influenza virus	$4.1 \times 10^6$	—	—	*
MM Poliomyelitis	$4.1 \times 10^6$	—	—	*

\* Non-irradiated was virulent; irradiated was non-virulent

## CONCLUSIONS

Studies on the cold sterilization of heat-sensitive pharmaceuticals, foods, and other materials by ionizing radiations have been encouraging. Considerable advance has been made in this field since World War II, but much research still remains to be done. Developments are occurring every day with newer sources of radiations, more powerful than any in the past, which offer greater potentialities for sterilization by this means.

The application of information regarding the effects of ionizing radiations and the application of radiation-producing equipment to actual processing are still in their infancy, although many encouraging results have been obtained thus far. The interest currently

shown in investigations in this field of endeavors indicates how important cold sterilization might be to the food and drug industries, if its application to line operations should prove feasible. Realization of the potentialities in this field offers a challenge to all those who participate to bring about successful applications of cold sterilization.

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## 35

# PASTEURIZATION

## INTRODUCTION

FOOD spoilage is usually the result of microbial activity and the wholesomeness of a food depends largely upon the kind and number of micro-organisms it contains. The quality or grade of a food frequently is lowered by the presence of excessive numbers of bacteria. This is especially true of milk and milk products.

The technics employed in the food industry for the control of microbial life are, as a rule, not sufficiently exacting to meet the requirements for sterilization. Rather, they aim for the reduction of bacteria within the commercially accepted limits, without requiring excessive equipment, too much labor or injury to the quality of the product. This is in contrast to the complete destruction of all life which must be the object of surgical and bacteriological sterilization.

The term "pasteurization" takes its name from Louis Pasteur who first employed moderate heating as a method of controlling the contaminating wild yeasts and bacteria responsible for the spoilage of wine. He found that temperatures of 50° to 60° C, maintained for a few minutes, gave excellent results, and the adoption of this principle did much to save the wine industry of France. So successful was Pasteur in solving this problem that he was requested to study abnormal fermentations of beer and, in 1870, he found that similar treatment was applicable to beer. Thus, pasteurization was developed, not as a method of improving the safety and keeping quality of milk or of combating the spread of human disease, but as an aid in producing wine and beer of high quality. Today, we recognize pasteurization as a process of checking or delaying bacterial decomposition of food and other substances, by exposing them to heat in such a manner as to effect a partial destruction of the contained micro-organisms, leaving alive only those that are in the spore stage and others that, though they survive, bring about changes in the substance slowly or not at all.

Holder pasteurization was practiced to a limited extent before 1900, the standards set by certain boards of health being 150° to 155° F for 15 to 20 minutes. However, milk dealers objected strenuously to the process because of the reduction in creaming ability.

Theobald Smith (1899) established the thermal death point of the tubercle bacillus in milk and reported complete destruction when held at 60° C (140° F) for 15 minutes. Other observers, notably Russell and Hastings in 1900, and Rosenau (1908) confirmed Smith's findings. From these studies it was recommended that 140° F for 30 minutes be adopted as the standard for holder pasteurization.

**EARLY PASTEURIZATION OF MILK.**—Following Pasteur's successful researches on the "diseases of wine," a law was established by Denmark in 1898, compelling the heating of skim milk to a temperature of 85° C (185° F) before being fed to calves and hogs. This early regulation was designed to prevent the dissemination of tuberculosis and other diseases from infected cows and is recognized as the forerunner of commercial milk pasteurization. As inconsistent as it may appear, the early pasteurization of milk was to protect animals, not humans, from infection.

In 1886, Soxhlet recommended that all cows' milk fed to infants be boiled for at least 35 minutes in order to destroy contaminating germs. He developed a simple home pasteurizer consisting of a number of 5-ounce bottles and trays which were immersed in a water-bath. This method was widely adopted in Austria and Germany and was followed by a distinct lowering of the death-rate of bottle-fed babies.

Soon after this period, Caille of New York visited Germany and upon his return to this country advocated the adoption of Soxhlet's method. The philanthropist, Nathan Straus, used this method for the pasteurization of milk in the first infant milk depot in America, established in New York City in 1893. In the Straus plant, the milk was pasteurized by the holder process for 20 minutes at 75° C (167° F). With this impetus, it was not long until various methods of pasteurization were being employed, especially the "flash" or quick method in which milk is maintained at a high temperature for a very short period.

## PASTEURIZATION DEFINED

Various legal definitions of milk pasteurization have been adopted by municipalities and states. On the whole, these differ but little from the definition contained in the milk ordinance as

recommended by the U. S. Public Health Service (1940): "The terms 'pasteurization', 'pasteurized' and similar terms shall be taken to refer to the process of heating every particle of milk or milk products to at least 143° F (61.7° C) and holding at such temperature for at least 30 minutes, or to at least 160° F (71.1° C) and holding at such temperature for at least 15 seconds, in approved and properly operated equipment . . ." From the public health viewpoint, the object of pasteurization is to render milk safe for human consumption by destroying the various pathogenic organisms which it may contain, and to accomplish this with the least possible alteration to the physical, chemical and nutritional properties of the milk.

### EQUIPMENT USED IN THE HOLDING METHOD OF PASTEURIZATION

Most milk is pasteurized by heating to 61.7° C (143° F) and holding for 30 minutes. The pasteurizers used are carefully designed stainless steel tanks in which the milk is heated, as shown in Figure 69. The tanks are of double wall or jacketed construction through which the heating medium, hot water or steam, circulates. In operation, a film of tempered water, slightly warmer than the final temperature of the milk, is spread over the sides of the pasteurizing vessel through a spray ring at the top of the jacket and over the bottom through bottom spray jets providing efficient and rapid heat transfer. The water thus used is drawn out of the bottom of the jacket by a close coupled, vertically mounted centrifugal pump and discharged through a steam mixing chamber back to the spray ring and bottom spray jet. The milk is agitated either by paddles or a propeller to obtain thorough mixing and uniform heating and to prevent the formation of a pellicle of partially dried milk or "milk skin."

*Foam Heaters.*—Foam is broken down and the air above the surface of the milk is heated by admitting a sufficient quantity of clean, dry steam into the air space during the pasteurizing process. The prevention of a pellicle and the control of foam are highly important because organisms can survive in either the pellicle or the foam. Milk sanitarians agree that the space above the milk should be moist and at least 5° F hotter than the milk. The necessity of additional heat and moisture in the air space above the surface of the milk is difficult for many operators to understand and unless the inspector is constantly on the alert, this feature may be neglected.



*Thermometers.*—Pasteurizing vats are equipped with indicating thermometers which serve as a guide for the operator and recording thermometers which provide a written record of the time the milk was heated and the temperatures employed. Modern recording thermometers are also designed to show the time the outlet was opened to draw off the pasteurized milk from the vat. Although these devices are somewhat expensive, they are nevertheless essential for the protection of the consumer.



Fig. 69.—Multiple-process holding pasteurizers at Heatherwood Farms, Lansing, Mich. (Courtesy of The Creamery Package Co., Chicago, Ill.)

## HIGH-TEMPERATURE SHORT-TIME PASTEURIZERS

The modern high-temperature short-time pasteurizers which quickly heat milk to at least  $71.1^{\circ}\text{C}$  ( $160^{\circ}\text{F}$ ) and positively hold every particle of such milk at this temperature for at least 15 seconds, without overheating, overholding, or injury to the cream line or flavor are precision instruments. Three general types of high-temperature short-time pasteurizers are in common use. The plate type, probably the most popular design, (Fig. 70), consists

of a series of thin stainless steel plates hanging in a suitable frame or press and so assembled that hot water flows between alternate plates while thin sheets of milk flow in the opposite direction. These plates are constructed in such a manner that they may be easily cleaned and sterilized when removed from the press.

A less popular type of pasteurizer is the internal tube heat exchanger. This equipment heats the milk by pumping it rapidly through small pipes contained in larger pipes through which hot water is pumped in the direction opposite to the flow of the milk.

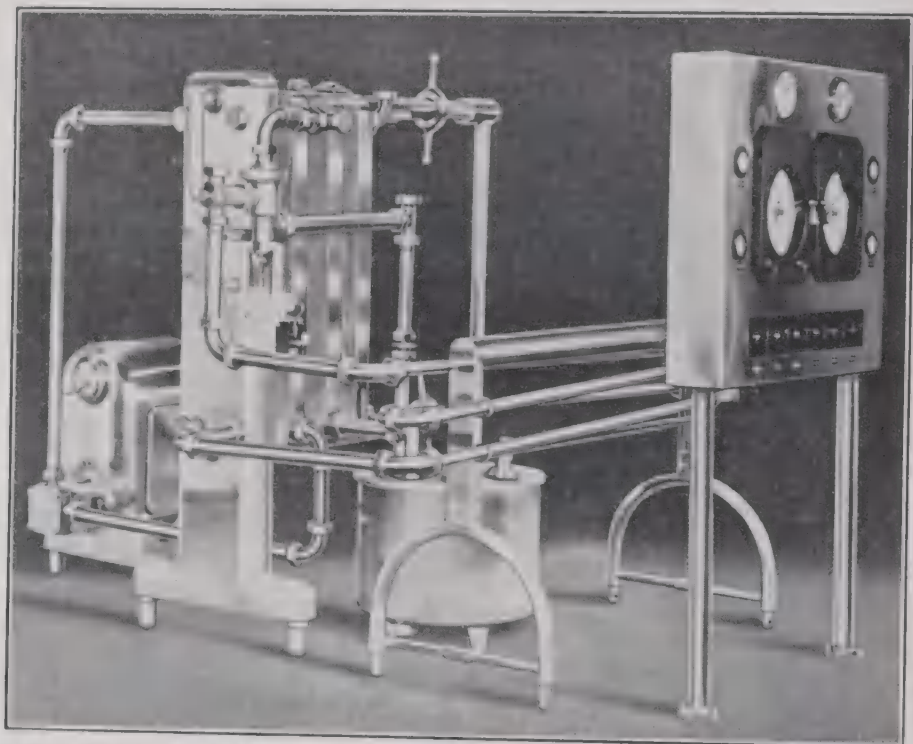


Fig. 70.—Superplate short-time pasturizer. (Courtesy of Cherry-Burrell Corp., Chicago, Ill.)

Electric pasteurizers are also used to some extent. With this process the electrical resistance of the milk results in a liberation of heat actually within the milk substance. The time of exposure and the temperature are accurately controlled by automatic devices and a flow diversion valve affords protection against the passage of under-pasteurized milk.

In general, the necessary automatic controls make high-temperature short-time pasteurizers rather costly and they are not always practical for small plants. In large plants, the high-temperature short-time pasteurizers are in favor because of the large capacities, compact design requiring minimum floor space and economy of operation and maintenance.

## COMPARISON OF HIGH-TEMPERATURE SHORT-TIME AND HOLDING METHODS

Either high-temperature short-time pasteurization for 15 seconds at 160° F or holding milk for 30 minutes at 143° F will destroy the bacteria responsible for milk-borne diseases. From a public health standpoint, either method is safe. The time-temperature equivalents necessary to bring about an equal degree of bacterial destruction in milk, as stated by Wilson (1942), are given in Table 130. This shows that the same degree of bacterial destruction can be brought about by exposing the milk to a temperature of 140° F for 1 hour, or to a temperature of 149° F for 6 minutes, or to a

TABLE 130.—TIME-TEMPERATURE EQUIVALENTS NECESSARY TO BRING ABOUT AN EQUAL DEGREE OF BACTERIAL DESTRUCTION IN MILK (FROM WILSON, 1942)

<i>Temperature</i>		
<i>° C</i>	<i>° F</i>	<i>Time</i>
60	140.0	63 minutes
62	143.6	25 "
63	145.4	16 "
64	147.2	10 "
65	149.0	6 "
66	150.8	4 "
70	158.0	38 seconds
72	161.6	15 "
74	165.2	6 "
76	168.8	2.4 "
80	176.0	0.4 "
85	185.0	0.04 "

temperature of 161.6° F for 15 seconds. The author has stated, however, that the time values given in this table for the upper ranges of temperature are only approximate, and are of greater academic than practical significance.

The holding process is frequently manually controlled and thus subject to the limitations of human error; the high-temperature short-time pasteurizer depends almost entirely upon automatic controls to insure proper treatment and these controls might be subject to mechanical failure. The phosphatase test is used to check the completeness of pasteurization by the holding method, and while this test is also useful in checking the high-temperature short-time method, there is evidence that at higher temperatures the enzyme may be inactivated before all pathogens are killed.

Economy of first cost and cost of operation, the effect upon the flavor of the milk and upon the volume of cream which rises in the pasteurized milk, and the keeping qualities of the pasteurized



milk are additional factors taken into consideration in selecting one method over the other.

*Reduction of Viable Bacteria by Pasteurization.*—Generally speaking, pasteurization reduces the total number of bacteria by 97 to more than 99 per cent, although wide deviations from these estimations are known to occur. Average figures which would be applicable under all conditions are difficult to obtain and might be misleading, since some types of micro-organisms multiply at the temperature used in the holding method and are not appreciably affected by the high-temperature, short-time method. In contrast, practically all of certain other types of bacteria succumb to pasteurization.

Those organisms which gain access during milking are likely to be dust-borne spores resistant to pasteurization, while those which are added as the result of inadequate sterilization of the milk equipment are likely to be destroyed by pasteurization. However, thermophilic or thermoduric organisms may contaminate milk utensils, especially when they are "sterilized" by hot water, and these organisms are known to be resistant to pasteurizing temperatures.

In observations of 44 pasteurizing plants supplying milk to the St. Louis market, McCulloch (1945) noted greater reductions in the bacterial counts than generally are recorded. During the summer of 1934, the mixed raw milk before pasteurization not infrequently contained from 10,000,000 to 20,000,000 organisms per ml, yet when pasteurized at 143.5° F for 30 minutes, counts as low as 200 to 1000 were not unusual, which would give a bacterial reduction of 99.99 to 99.999 per cent. In most instances this milk had been hauled many miles without cooling, and the organisms were in the logarithmic phase of growth.

### TERMINAL HEATING OF INFANT FORMULAS

Milk and milk mixtures are excellent culture media for bacteria and consequently they are preeminently fitted to convey the germs of infectious disease. One of the more common causes of outbreaks of diarrhea in hospital nurseries is believed to be contamination of milk preparations and equipment used in the feeding of newborn infants. For example, Rubinstein and Foley (1947) reviewed 19 outbreaks of epidemic diarrhea of the newborn as reported to the Massachusetts Department of Health from 1935 to 1945. Their investigations revealed many inadequacies and almost unbelievable breaks in nursing and formula-making technics. Laboratory examination of formulas, nipples and other utensils, showed heavy bacterial contamination.

Experience has demonstrated that it is not advisable for a hospital to depend entirely upon the efficiency of commercial pasteurization of milk as delivered from local dairies for the feeding of infants. In fact, it would seem logical to consider that a disease in the community may be the possible source of a nursery infection due to a contaminated milk supply. Such a condition was reported by Ensign and Hunter (1946) in an epidemic of diarrhea among 24 newborn infants in which there were 9 deaths. The infants were apparently secondarily infected by mothers and nurses who drank the contaminated milk in the hospital.

*Approved Methods of Terminal Heating.*—Terminal heating of formulas should be universally adopted in hospitals. It is a precaution which under practical conditions of operation can assure bacteriologically safe milk mixtures for infants. The total heat treatment must be adequate for the destruction of pathogens. Sterility of the formula is highly desirable but not always obtainable by presently accepted methods of terminal heating. The amount of heat that can be applied is limited by the fragile character of milk which, if heating is too severe, will suffer both physical and nutritional damage.

At the present time public health authorities and other regulatory bodies recognize two methods for terminal heating of formulas—the non-pressure (flowing steam) process at 100° C (212° F) for 25 to 30 minutes and the pressure steam method at 110° C (230° F) (7 pounds) for 10 minutes. When properly conducted either method can be depended upon to render formulas bacteriologically safe and uniform in quality. However, the pressure method is somewhat more troublesome than the non-pressure (flowing steam) process because it requires careful mechanical control and attention on the part of the operator during the sterilizing cycle. Too rapid exhaust of the steam from the sterilizer frequently results in boiling of the formulas. When this occurs, the nipples may be blown off the bottles, the holes in the nipples clogged with dried milk. If formulas boil over there is the likelihood of coagulated milk gaining access to the outlet valve and then clogging the chamber drain line of the sterilizer. However, if the operator exercises the proper care and attention and allows 10 to 15 minutes for slow exhaust of the pressure from the sterilizer such objections can be overcome.

Terminal heating by the non-pressure (flowing steam) method can also be performed in the autoclave, providing it is properly piped and valved so as to permit atmospheric steam at a temperature of 100° C (212° F) to circulate freely through the chamber. With a dual range regulator on the autoclave it is also possible to

use the same chamber for pressure steam sterilization at 121° C (250° F) of empty bottles, nipples, dry goods and other supplies common to the formula room and nursery.

In general, it can be expected that terminal heating by either of the above-mentioned methods will produce formulas which are either sterile or sufficiently low in count of non-pathogenic organisms to meet the accepted standard of quality. The number of surviving organisms in the formula should not exceed a count of 25 per ml by the "standard plate" method.

*Formula Room Technic.*—Even though there may exist some diversity of opinion with respect to the various details of formula room procedure, it is generally recognized that the combined application of a strictly clean technic with an approved method of terminal heating offers the greatest promise of success in producing formulas that are uniform in quality and bacteriologically safe. The procedure given below is typical of standard practice in many hospital formula rooms:

1. Before performing any duty in the preparation room the nurse employs the conventional 3-minute scrub.
2. All bottles, nipples and utensils are thoroughly washed in hot water containing an effective detergent, rinsed, and then sterilized by pressure steam at 250° F for 10 minutes.
3. The formula ingredients (milk, sugar or modifier) and water are then placed in sterile containers, mixed, and the formula dispensed into previously washed sterile bottles.
4. Sterilized nipples are then attached to the bottles and each nipple completely covered with a paper cap, extending well over the shoulder of the bottle and held firmly in place with rubber band.
5. Following preparation of the assembled formulas, they are immediately placed in the sterilizer and subjected to non-pressure (flowing) steam at a temperature of 212° F for 30 minutes.
6. The formulas are then allowed to cool at room temperature for 1 to 2 hours. Finally, they are transferred to the refrigerator maintained at a temperature of 40 to 45° F where they remain until feeding time.

With the technic outlined above for terminal heating of formulas in bottles with nipples attached, it might be argued that pre-sterilization of bottles, nipples and utensils is an unnecessary step and that thorough washing of the equipment should suffice as a preparatory measure. Theoretically that is true. However, one cannot always be sure of the thoroughness of the washing process, especially if milk has been permitted to dry on the inside of bottles



and nipple surfaces. Even then it is acknowledged that neither pre-sterilization of bottles and utensils nor terminal heating of the formulas can altogether compensate for poor cleaning. Finley, Smith and Louder (1948) showed experimentally that formula bottles used day after day without thorough cleaning eventually can produce contaminated formulas in spite of a terminal heat treatment of 230° F for 10 minutes.

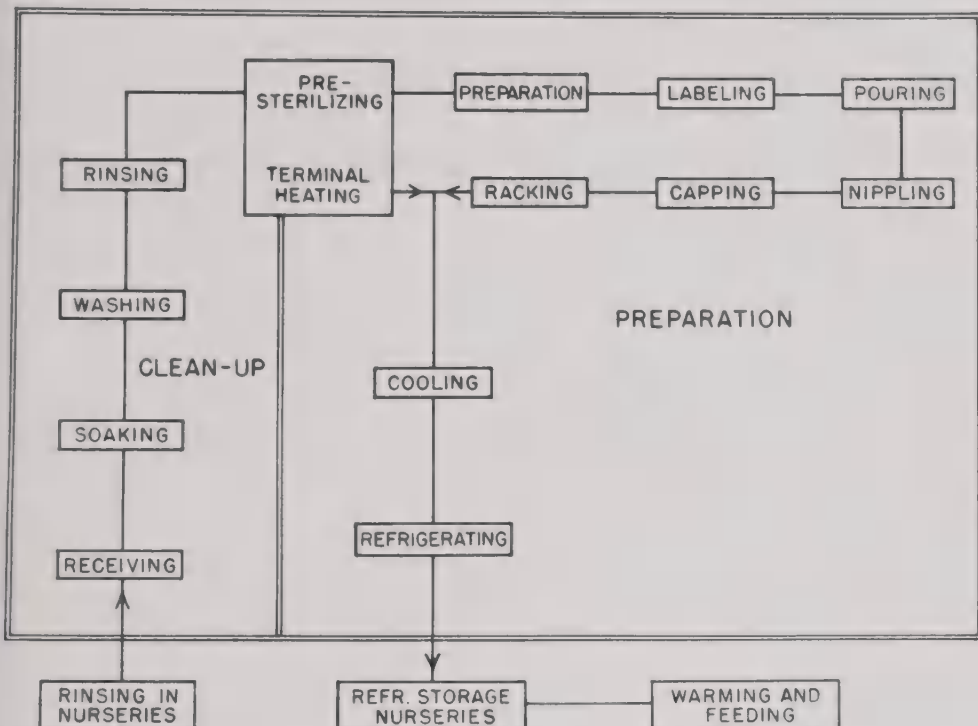


Fig. 71.—Formula room flow chart. The continuity of procedure is based upon positive segregation of the clean-up area from the formula preparation area. The only means of communication between the two sections is through a double door sterilizer. (Courtesy American Sterilizer Co.)

The extra precaution of pre-sterilization of bottles, nipples and utensils is considered by many to be good practice if for no other reason than to prevent the entrance of potentially contaminated equipment into the formula preparation room. (See Flow Chart Fig. 71). Also, when special formulas are required that cannot be subjected to terminal heating, the feature of pre-sterilization as a part of the standard formula procedure makes it unnecessary to set up a special technic. Since authorities are in agreement that nipples especially should undergo pre-sterilization as an extra precaution, it is only logical that the bottles and utensils should likewise be pre-sterilized and thereby effect a standard procedure.

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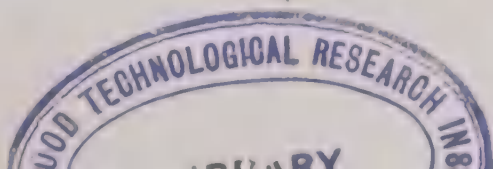
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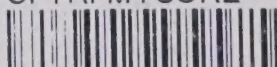
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